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X. ON THE OXIDATION OF AMINO-ACIDS AND OF RELATED SUBSTANCES WITH CHLORAMINE-T.

By HENRY DRYSDALE DAKIN.

(*Report to the Medical Research Committee.*)

(*Received April 24th, 1917.*)

Reference has already been made [1916, 1, 2] to the oxidation of certain amino-acids with hypochlorite and with chloramine-T. The latter term is the abbreviated name for sodio-*p*-toluenesulphochloroamine, a substance which appears to constitute a useful addition to the small class of neutral oxidising agents. These experiments seemed worth extending, partly because interesting products of oxidation were likely to be met with and also as preliminary to a study of the mode of antiseptic action of substances of the chloroamine group. In the case of glycine, alanine, leucine, and α-aminophenylacetic acid it was found that the first product of oxidation, using one molecule of oxidising agent, was an aldehyde, as is usually the case with hypochlorite oxidation, as shown by Langheld. On using two molecules of chloramine-T for the oxidations, nitriles were formed, sometimes in good yield. With histidine, no aldehyde[1] could be isolated, but an excellent yield of cyanomethylglyoxaline was obtained.

The present communication deals especially with the oxidation of the neutral salts of glutamic, aspartic, and methylaspartic acids and asparagine, valine and isoleucine.

Glutamic acid may be considered first since the reaction is less complex than with the other substances.

On acting on a dilute aqueous solution of the mono-sodium salt of glutamic acid (1 mol.) with chloramine-T (1 mol.) an excellent yield of the semi-aldehyde of succinic acid (β-aldehydopropionic acid) is obtained. It is most readily separated and identified by means of its nitrophenylhydrazone, but the substance itself may be isolated and distilled although with considerable

[1] It would appear that Langheld's observations on the formation of iminazoleacetaldehyde by the action of hypochlorite on histidine require confirmation.

loss, as described by Harries and Alefeld [1909]. The reaction may be expressed as follows:

$$COOH \cdot (CH_2)_2 \cdot CH(NH_2) \cdot COOH \rightarrow COOH \cdot (CH_2)_2 \cdot CH(NHCl) \cdot COOH \rightarrow COOH \cdot (CH_2)_2 \cdot CHO + NH_4Cl + CO_2.$$

Using two molecules of chloramine-T to one of the glutamic salt, the reaction takes a different course with formation of large quantities of β-cyanopropionic acid, but only negligible traces of succinic acid. The formation of β-cyanopropionic acid is noteworthy since the preparation of this acid has long been unsuccessfully attempted; the methods which would ordinarily be chosen for preparing the substance not having given the desired result.

β-Cyanopropionic acid is a strong monobasic acid, which is readily obtained in crystalline form and with a satisfactory yield. It is likely that it is formed as the result of the decomposition of a dichloroamino-acid, although the latter unstable substance was not isolated.

$$COOH \cdot (CH_2)_2 \cdot CH(NH_2) \cdot COOH \rightarrow COOH \cdot (CH_2)_2 \cdot CH(NCl_2) \cdot COOH \rightarrow COOH \cdot (CH_2)_2 \cdot CN + CO_2 + 2HCl.$$

The constitution of the cyano-acid was definitely fixed by its quantitative conversion into succinic acid on treatment with concentrated hydrochloric acid and by its reduction to γ-aminobutyric acid by sodium and alcohol. The latter substance was obtained in very small yield but its formation is of interest since it has been obtained by the bacterial decomposition of glutamic acid.

$$COOH \cdot CH_2 \cdot CH_2 \cdot CN \nearrow COOH \cdot CH_2 \cdot CH_2 \cdot COOH$$
$$\searrow COOH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot NH_2.$$

Aspartic acid. The oxidation of aspartic acid, or rather its neutral sodium salt, proved to be a more complicated process. On adding chloramine-T in the proportion of one, two or three molecules to sodium aspartate (1 mol.) in 1–5 % aqueous solution, an immediate reaction took place with precipitation of toluenesulphonamide and liberation of ammonia and carbon dioxide. On heating the filtered solution with phenylhydrazine or semicarbazide or with aromatic orthodiamines, derivatives of glyoxal were obtained in large amounts. The best yields were obtained when between two and three molecular proportions of the chloramine-T were used and with the higher limit the yield of carbon dioxide closely approached two molecules. This carbon dioxide undoubtedly came from the two terminal carboxyl groups, leaving the two adjacent carbon groups to furnish the glyoxal derivatives. The apparent direct oxidation of the (CH_2) group to CHO seemed surprising,

and eventually another explanation was found. On oxidising asparagine with chloramine-T, among other products a substance was isolated which proved to be dichloroacetamide. This at once indicated the probability that the hydrogen of the (CH_2) group in aspartic acid, on oxidation with chloramine-T was replaced by chlorine. This proved to be the case and the substance yielding the glyoxal derivatives was identified as dichloroacetaldehyde. Similarly it was found that methylaspartic acid,

$$COOH \cdot C(NH_2)(CH_3) \cdot CH_2 \cdot COOH,$$

on oxidation gave derivatives of methylglyoxal which originated from dichloroacetone.

It appears probable that the half aldehyde of malonic acid is the first product of the oxidation of sodium aspartate with chloramine-T and that the former substance in neutral or alkaline solution undergoes chlorination at the methylene group, giving the half aldehyde of dichloromalonic acid which then loses carbon dioxide to give dichloroacetaldehyde.

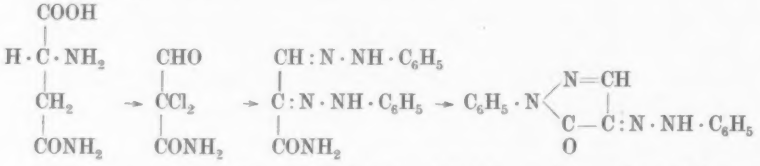
$$\begin{array}{cccccc} COOH & & & & & \\ | & & & & & \\ CHNH_2 & CHO & & CHO & & \\ | & | & & | & & \\ CH_2 & \rightarrow CH_2 & \rightarrow & CCl_2 & \rightarrow & CHO \\ | & | & & | & & | \\ COOH & COOH & & COOH & & CHCl_2 \end{array}$$

The ready chlorination of the half aldehyde of malonic acid is not surprising. Apparently chloramine-T chlorinates free acetaldehyde much less readily than is observed in the present reaction, so that it appears improbable that acetaldehyde is a primary product of oxidation which subsequently undergoes chlorination, although the formation of acetaldehyde from aspartic acid by oxidation with sodium hypochlorite or hydrogen peroxide is well established. Unlike glutamic and many other amino-acids, which yield nitriles on oxidation with two molecules of chloramine-T, no indications were obtained of the presence of cyanoacetic acid among the oxidation products of aspartic acid. On the other hand, the presence of a small quantity of a volatile compound, soluble in ether, giving a strong diazo-reaction, was noted. This substance has not yet been identified but appears to be a glyoxaline derivative. The same compound appears to be formed by the interaction of dichloroacetaldehyde and ammonia, and as both of these latter substances are formed in the oxidation of sodium aspartate by chloramine-T, its formation is in a measure accounted for. The action of ammonia on dichloroacetaldehyde invites further study.

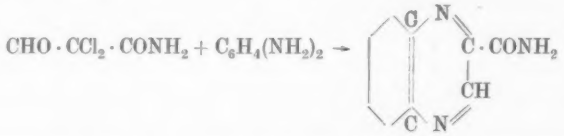
Asparagine. On oxidising aqueous solutions of asparagine with chloramine-T, a reducing substance is formed which, on treatment with phenylhydrazine and other bases, gives derivatives of mesoxalic semi-aldehyde. The substance was not isolated in the pure state but was characterised by a number of derivatives, all of which indicated that its constitution might be represented by some such formula as $CHO \cdot CCl_2 \cdot CO \cdot NH_2$, *i.e.* the semi-aldehyde of dichloromalonamide. In addition, as already mentioned, a considerable quantity of dichloroacetamide was isolated from the oxidation products. Neither formaldehyde nor acetaldehyde was found in appreciable amounts.

On treating solutions containing the semi-aldehyde of dichloromalonamide with phenylhydrazine acetate in the cold, an oily precipitate is first formed due to the formation of the mono-hydrazone, but on warming, chlorine is removed and the whole is converted into a finely crystalline compound, namely the amide of mesoxalic semi-aldehyde *bis*-phenylhydrazone. On heating the latter compound with dilute hydrochloric acid, it is converted into 4-benzeneazo-1-phenyl-5-pyrazolone already obtained by Knorr and by Will.

The changes may be indicated as follows:



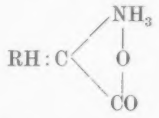
Another reaction of the semi-aldehyde of dichloromalonamide, which may be of practical value, is the fact that it reacts with various orthodiamines to give the amides of quinoxaline-carboxylic acids. These amides as well as many of the corresponding mono-carboxylic acids appear to have been hitherto inaccessible.



Reference must be made here to Langheld's observations on the action of sodium hypochlorite upon asparagine. He states that the product of the reaction is the hitherto unknown half-aldehyde of malonic acid which he did not isolate but characterised as a phenylhydrazone melting at 239–240°. Satisfactory analyses for this compound were recorded by Langheld (see

experimental section) but as a matter of fact the compound is the *bis*-phenylhydrazone of mesoxamide semi-aldehyde described above. Direct comparison of the compounds, as well as their mixed melting point and conversion into 4-benzeneazo-1-phenyl-5-pyrazolone prove this conclusively. Langheld's description of the half aldehyde of malonic acid and its hydrazone must therefore be abandoned.

It is a somewhat striking fact that so far as at present observed, chloramine-T does not react at all readily with amino-acid esters. This is the more surprising in view of the pronounced basic properties of the esters. It is possible that chloramine-T reacts with the cyclic betaine form of amino-acids,



rather than with the carboxylic form.

In the experimental portion are included experiments demonstrating the formation of isobutyric aldehyde from valine and of methylethylacetaldehyde from isoleucine on oxidation with chloramine-T. These reactions are typical of those previously encountered and present nothing essentially new.

An addendum to the experimental portion gives an account of an interesting substance obtained during the oxidation of carbohydrates by chloramine-T together with a number of its derivatives. The substance proved to be the hitherto unknown benzaldehyde-*p*-sulphonamide. The production of this compound is rather curious. It appears to be produced by the action of chloramine-T upon *p*-toluenesulphonamide, the latter substance being formed by the oxidising action of chloramine-T upon the sugar. The apparent anomaly is thus presented of chloramine-T oxidising its own reaction product. The formation of benzaldehyde-*p*-sulphonamide appears worthy of record since this substance could not be readily synthesised by the usual methods. The other products derived from the glucose are complicated and need further study, although small quantities of saccharic acid have been definitely identified, while little gluconic acid is found, although pentoses are present in noteworthy amount.

Experimental Part.

Oxidation of glutamic acid.

Formation of β-aldehydopropionic acid. This substance is readily formed when neutral sodium glutamate is treated with one molecular proportion of chloramine-T. Glutamic acid (1·47 g.) was dissolved in 100 cc. of hot N/10 sodium hydroxide and the solution cooled to air temperature. Solid chloramine-T (2·9 g.) was next added, which readily dissolved, *p*-toluenesulphonamide being then rapidly precipitated. The reaction did not readily proceed to completion in the cold but on gently warming to 50° a steady evolution of ammonia and carbon dioxide took place and active chlorine, tested for with potassium iodide and acetic acid, rapidly disappeared. The solution was then chilled and the sulphonamide removed by filtration. The filtrate on treatment with nitrophenylhydrazine (1·7 g.) dissolved in hot dilute hydrochloric acid gave 2·1 g. of the nitrophenylhydrazone of β-aldehydopropionic acid compared with a theoretical yield of 2·37 g. The substance was purified by recrystallisation from boiling water and crystallised in platelets which when rapidly heated melted at 185–187°. On analysis the substance was found to contain 17·9 % nitrogen (Dumas) compared with a calculated value of 17·7 for $C_{10}H_{11}O_4N_3$. In the original paper by Harries and Alefeld [1909] this compound was described as melting at 158° and this value later was corrected by Alefeld [1909] who gave 175°.

A second oxidation was carried out with larger quantities of glutamic acid (5·41 g.), but otherwise under similar conditions. After filtering off the sulphonamide, the solution was concentrated under reduced pressure and the filtered concentrated solution acidified with dilute sulphuric acid and extracted thoroughly with ether in a continuous extraction apparatus. On distilling off the ether and distilling the residue under a pressure of 2 mm. a small amount of the oily β-aldehydopropionic acid was obtained as described by Perkin [1899] and by Harries and Alefeld, but by far the greater part of the aldehyde had undergone oxidation during the rather protracted manipulations, so that much succinic acid, m.p. 185°, was obtained in the second fraction. The aldehyde was characterised as nitrophenylhydrazone, m.p. 185–187°, and semicarbazone, m.p. 177–178°.

Formation of β-cyanopropionic acid. Glutamic acid (7·35 g.) was dissolved in 250 cc. 0·2 N sodium hydroxide and chloramine-T (28·4 g. = 2 mols.) added to the cooled solution. The mixture was kept at 35° for a couple of hours, then cooled and filtered from the precipitated sulphonamide. The

filtrate was thoroughly extracted with ether in a continuous extraction apparatus to remove remaining traces of sulphonamide, then acidified with sulphuric acid and again extracted to separate the cyano-acid. The second ether extract on evaporation gave 4·75 g. of crude cyanopropionic acid which readily solidified on standing a short time. The substance was almost pure save for a trace of succinic acid which was removed by dissolving the dry residue in a little absolute alcohol, then adding chloroform and a little petroleum ether. The succinic acid was readily precipitated and filtered off and the filtrate on concentration gave a crystalline mass of pure β-cyanopropionic acid which was purified by washing with a little chloroform. The acid is colourless, crystallises in stout prisms and melts at 48–50° without decomposition.

Analysis: 0·1587 g.; 0·2817 g. CO_2; 0·0733 g. H_2O.
0·1058 g.; 13·2 cc. moist N at 20° and 762 mm.

	C	H	N
Found %	48·3	5·1	14·2
Calculated for $C_4H_5O_2N$ %	48·5	5·1	14·1

β-Cyanopropionic acid is a strong acid, freely soluble in most organic solvents, except petroleum ether, and extremely soluble in water, although not deliquescent. 0·111 g. of the acid required 11·1 cc. of N/10 sodium hydroxide to neutralise to phenolphthalein, compared with a calculated value of 11·2 cc. The sodium salt is very soluble in water and aqueous solutions of it are not precipitated by lead acetate, silver nitrate or mercuric chloride.

On evaporation of β-cyanopropionic acid with concentrated hydrochloric acid on the water-bath, it is quantitatively converted into succinic acid, m.p. 185°. The succinic acid was obtained pure on washing the residue with a little cold water to remove ammonium salts and then crystallising from dilute hydrochloric acid.

The reduction of β-cyanopropionic acid was carried out by dissolving 5 g. of the acid in 75 cc. of alcohol and rapidly adding an excess of metallic sodium (7 g.). The mixture was diluted with water, neutralised with dilute sulphuric acid and the alcohol evaporated off on the water-bath. The aqueous solution was then precipitated with phosphotungstic acid in 5 % sulphuric acid. A fine, heavy precipitate was obtained, which after standing was decomposed in the usual way with barium hydroxide. The free γ-aminobutyric acid obtained on evaporation after removing excess of barium hydroxide, was dissolved in hot alcohol and then precipitated with chloroplatinic acid, when the characteristic orange yellow platinum salt, melting

at 218–220°, was obtained. (Pt 31·4 %, calculated 31·7 %.) The yield of γ-aminobutyric acid was very poor, amounting to about 5 % of the theoretical amount. It is not unlikely that the conditions of the reduction might be advantageously modified.

Oxidation of aspartic acid.

Aspartic acid was neutralised with 0·1 or 0·2 normal sodium hydroxide and oxidised with chloramine-T, as already directed for glutamic acid, but no essential difference in the nature of the products was noted when either one, two or three molecular proportions of the oxidising agent were used. Quantitative estimations of the carbon dioxide formed in the process showed that 1 gram-molecular proportion of sodium aspartate gave 0·65, 1·43 and 1·8 molecular proportions of carbon dioxide when treated respectively with 1, 2 and 3 gram-molecules of chloramine-T. In each case a volatile aldehyde was present, which proved to be chiefly, if not exclusively, dichloroacetaldehyde.

On treating the filtrates from the toluenesulphonamide with phenylhydrazines either before or after distillation, glyoxal hydrazones derived from dichloroacetaldehyde were readily obtained in fair yield. For example, 1·33 g. aspartic acid was dissolved in 100 cc. 0·1 N sodium hydroxide and treated with 2·84 g. (1 mol.) chloramine-T. The reaction was prompt but active chlorine persisted for some time at room temperature so the mixture was incubated at 35° for half-an-hour. The mixture, which had an odour suggestive of a little isonitrile, was then chilled and the toluenesulphonamide filtered off. On warming the filtrate with excess of phenylhydrazine acetate 0·7 g. of glyoxal *bis*-phenylhydrazone was obtained. Similar experiments with two and three molecular proportions of chloramine-T gave yields of 1·4 and 1·2 g. respectively.

The glyoxal *bis*-phenylhydrazone was crystallised from alcohol and melted at 170–171°. It was free from chlorine and gave the following results on analysis:

0·1025 g.; 0·2650 g. CO_2; 0·0540 g. H_2O.

	C	H	N
Found	70·5	5·85	23·5
Calculated for $C_{14}H_{14}N_4$ %	70·6	5·88	23·5

On similar treatment with *p*-bromophenylhydrazine hydrochloride, glyoxal *bis*-bromophenylhydrazone melting at 240° was obtained, while *p*-nitrophenyl-

hydrazine hydrochloride gave the very characteristic glyoxal *bis*-nitrophenylhydrazone crystallising from pyridine in scarlet needles melting at about 315°.

The dichloroacetaldehyde was also converted into glyoxal *bis*-semicarbazone on digesting the aldehyde solution with excess of semicarbazide hydrochloride and sodium acetate. This reaction has already been observed by Kling [1909]. The product did not melt at 270° and was identical with the substance prepared directly from glyoxal.

Finally, the dichloroacetaldehyde was converted into β-dichlorolactic acid as follows: aspartic acid (13·3 g.) was neutralised with soda and oxidised with chloramine-T (56·8 g. = 2 mols.) as before described. After filtration, the ammonia formed in the reaction was neutralised by addition of acetic acid and the mixture distilled. The distillate was treated with hydrocyanic acid and hydrochloric acid according to the directions of Grimaux and Adam [1880], and after standing over night the nitrile was hydrolysed by boiling. On extraction with ether β-dichlorolactic acid crystallising in platelets and melting at 76–77° was readily obtained.

It is noteworthy that the distillate from the aspartic acid oxidations, especially when free ammonia was not neutralised, gave intense red colour reactions with diazobenzenesulphonic acid in sodium carbonate solution. The same phenomenon was observed when pure dichloroacetaldehyde was distilled with aqueous ammonia, and it seems probable that some glyoxaline synthesis had occurred. The constitution of the compound remains to be determined.

Oxidation of asparagine.

Asparagine was oxidised with chloramine-T in aqueous solution, no addition of alkali being necessary. The reaction commences rapidly at room temperature, with precipitation of toluenesulphonamide. When one or two molecular proportions of chloramine-T are used, all trace of active chlorine disappears on warming the mixture to 30° for about half-an-hour, but with three molecules of chloramine-T the reaction for active chlorine usually persists for a long time even though unchanged asparagine may be present in solution. The products of the reaction are ammonia, carbon dioxide, dichloroacetamide and a non-volatile aldehyde which was not obtained pure, but which is probably the semi-aldehyde of dichloromalonamide,

$$CHO \cdot CCl_2 \cdot CONH_2,$$

from which a variety of derivatives were obtained. The latter will be first described. About 25 % of the theoretical amount of the aldehyde, as judged

by its derivatives, was formed when one molecule of chloramine-T was used, about 50 % with two molecules, less with three and almost none with four. The yield of carbon dioxide was about 0·3 molecule for each molecule of oxidising agent.

Mesoxamide semi-aldehyde bis-*phenylhydrazone*,

$$CH : (N \cdot NHC_6H_5)C : (N \cdot NH \cdot C_6H_5) \cdot CONH_2.$$

Asparagine (6·6 g.) was dissolved in hot water (150 cc.), the solution cooled to room temperature and chloramine-T (28·4 g.) added. The reaction went smoothly, and after standing for half-an-hour at 30°, all active chlorine had disappeared. The mixture was cooled and the precipitated sulphonamide removed by filtration and the filtrate mixed with phenylhydrazine (10 g.) dissolved in excess of acetic acid. For a few moments there was no precipitation, then an unattractive-looking oil began to separate in the cold, but on warming on the water-bath, a finely granular reddish precipitate was formed. The hydrazone was filtered off and crystallised from boiling ethyl acetate. It forms light yellow prisms and needles melting sharply at 250–252°. The yield of hydrazone was 4·5 g.

Analysis: 0·1210 g.; 0·2841 g. CO_2; 0·0587 g. H_2O,
0·1500 g.; 33·2 cc. moist N at 19° and 750 mm.

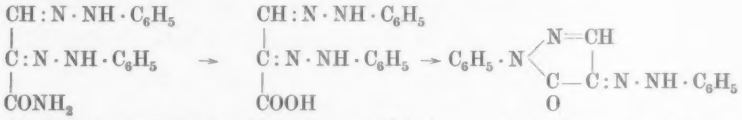
	C	H	N
Found %	64·0	5·38	25·0
Calculated for $C_{15}H_{15}ON_5$ %	64·1	5·32	24·8

The hydrazone is very soluble in warm glacial acetic acid, and moderately soluble in alcohol, ethyl acetate and acetone.

This substance is identical with the compound obtained by Langheld [1909] by the action of sodium hypochlorite upon asparagine to which he erroneously assigned the constitution of a mono-phenylhydrazone of the unknown amide of malonic semi-aldehyde. Langheld gave the melting point of the compound as 239–240° and gave analyses supporting his hypothesis. The calculated values for $C_9H_{11}N_3O_2$ are C 61·04 %, H 6·21 %, N 23·75 %, while Langheld found C 60·81 %, H 5·95 %, N 24·05 %. However, direct comparison of the product obtained according to Langheld's description and the substance above described proved their identity and the agreement of Langheld's values for the carbon and hydrogen with the mono-hydrazone, remains unexplained. The melting point of the product obtained according to Langheld's description was 250–252° and was unchanged on mixing with

the *bis*-phenylhydrazone above described and on elementary analysis his compound gave results practically identical with those for the latter substance. On treatment with acids both products gave 4-benzeneazo-1-phenyl-5-pyrazolone.

4-Benzeneazo-1-phenyl-5-pyrazolone. This substance was obtained on dissolving the above described *bis*-phenylhydrazone of the semi-aldehyde of mesoxamide in hot alcohol, adding about 5 % concentrated hydrochloric acid and boiling for two hours under a reflux condenser. On cooling, fine orange reddish needles melting at 150° were obtained, identical with the product obtained by Knorr [1888] and Will [1891] by the action of acetic anhydride or alcoholic hydrochloric acid on mesoxalic semi-aldehyde *bis*-phenylhydrazone. The reaction may be represented as follows:



bis-Phenylhydrazone of semi-aldehyde of mesoxamide — Mesoxalic semi-aldehyde *bis*-phenylhydrazone — 4-Benzeneazo-1-phenyl-5-pyrazolone

The constitution of the osazone from the oxidation products of asparagine is satisfactorily established by this reaction.

Mesoxamide semi-aldehyde bis-p-*bromophenylhydrazone.* This substance was obtained in the same way as the *bis*-phenylhydrazone by using an excess of *p*-bromophenylhydrazine hydrochloride at water-bath temperature. The solid product was filtered off, washed with water and a little warm alcohol. It was recrystallised from a mixture of glacial acetic acid and alcohol and formed golden yellow rosettes of needles melting sharply with decomposition at 274–275°. The substance is sparingly soluble in alcohol or ethyl acetate but readily soluble in acetic acid.

Analysis: 0·1782 g.; 24·2 cc. moist N at 15° and 752 mm.
Found ... ... 15·7 % N.
Calculated for $C_{15}H_{13}ON_3Br_2$ 15·9 % N.

Mesoxamide semi-aldehyde bis-p-*nitrophenylhydrazone.* This substance was prepared like the corresponding bromine derivative. 2 g. of asparagine when oxidised with chloramine-T (8·4 g.) gave, when treated with 3 g. nitrophenylhydrazine hydrochloride, 1·4 g. of the nitrophenylhydrazone. The compound was obtained as a deep scarlet red precipitate which was washed with alcohol and ether. It is very sparingly soluble in alcohol, ether, acetone, amyl alcohol or pyridine, but can be crystallised from boiling nitrobenzene.

It forms deep red prismatic needles melting at about 340°. A trace of the substance treated with sodium hydroxide and alcohol gives an intense blue solution, a reaction which appears to be characteristic of two adjacent nitrophenylhydrazine groups.

Analysis: 0·1500 g.; 30·5 cc. moist N at 21° and 750 mm.

Found	22·7 % N.
Calculated for $C_{15}H_{13}O_5N_7$	22·7 % N.

Mesoxamide semi-aldehyde bis-*semicarbazone.*

$$CH : (N \cdot NH \cdot CONH_2) \cdot C : (N \cdot NH \cdot CONH_2) \cdot CONH_2.$$

A part of the solution obtained from the oxidation of asparagine with two molecules of chloramine-T, as previously described, was concentrated under reduced pressure, then cooled and filtered from a little toluenesulphonamide. The filtrate was gently warmed with excess of semicarbazide hydrochloride, sodium acetate and a few drops of acetic acid. The semicarbazone was slowly precipitated as a heavy white crystalline substance. It is very sparingly soluble in alcohol or water and when recrystallised from either solvent is obtained in the form of fine felted needles melting at 240°.

Analysis: 0·1072 g.; 42·5 cc. moist N at 17° and 750 mm.

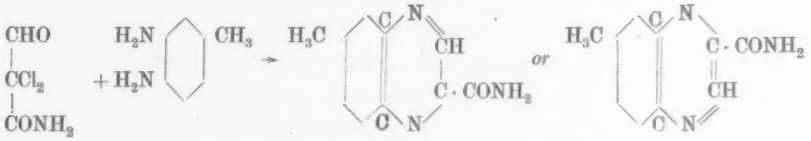
Found	45·1 % N.
Calculated for $C_5H_9O_3N_7$	45·5 % N.

Amide of 7-methylquinoxaline-2 or 3-carboxylic acid. The solution containing the oxidation products from 5 g. of asparagine prepared in the usual way, was treated with excess (3 g.) of 3 : 4-tolylene diamine. The mixture was acidified decidedly with acetic acid and then warmed on the water-bath for ten minutes. Sodium carbonate was then added to neutralise the acid and the precipitated quinoxaline was allowed to settle out. After a short time the product was filtered off, washed with a little alcohol to remove a small amount of resinous impurity and then crystallised from hot 90 % alcohol. The substance forms glistening plates which appear colourless under the microscope but which are slightly greyish brown in bulk. An alcoholic solution is light yellow in colour. The melting point of the recrystallised substance is 286–287°. The yield was poor, being only about 15 % of the calculated amount.

Analysis: 0·1117 g.; 0·2625 g. CO_2; 0·0483 g. H_2O.

	C	H
Found %	64·1	4·8
Calculated for $C_{10}H_9ON_3$ %	64·2	4·8

The formation of the quinoxaline compound may probably be represented as follows:



Other aromatic orthodiamines react similarly, but the products have not yet been characterised.

Separation of dichloroacetamide from oxidation products of asparagine.

Asparagine (5 g.) was dissolved in water (100 cc.) and oxidised at 20–25° with chloramine-T (25 g.). After two or three hours almost all active chlorine had disappeared and the solution was filtered from toluenesulphonamide and concentrated under reduced pressure to about 30 cc. A small additional separation of sulphonamide was filtered off. The filtrate was next extracted for several hours with ethyl acetate and the solvent was then removed under diminished pressure. Some of the aldehyde compound already discussed is found in the extract but much remains in the aqueous portion. The clear ethyl acetate extract, which often crystallised, was dissolved in a little hot water, treated with a little charcoal, filtered and concentrated slowly. Fine prismatic needles were thus obtained which were purified by dissolving in a little dry ether, filtering from a trace of insoluble matter and allowing the solution to evaporate spontaneously. In this way about a gram of large stout prisms of dichloroacetamide, melting at 96–98°, was obtained. The substance showed all the reactions characteristic of this compound and gave the following results on analysis: C 19·5, H 2·4, N 11·0, calculated for $C_2H_3ONCl_2$, C 18·9, H 2·34, N 10·9.

Oxidation of methylaspartic acid.

Methylaspartic ester was prepared by the action of potassium cyanide and ammonium chloride upon ethyl acetoacetate according to the directions of Zelinsky, Annenkoff and Kulikoff [1911]. The ethyl ester, as already mentioned, did not react at all readily with chloramine-T. The free acid was prepared by hydrolysing 25 g. of the ester with 40 g. barium hydroxide and 250 cc. of water. The mixture was heated two hours on the water-bath, barium salts were then removed exactly by sulphuric acid and the free acid crystallised from the concentrated aqueous solution.

The acid (7·35 g.) was neutralised with 50 cc. normal sodium hydroxide in 450 cc. of water, and chloramine-T (28·4 g.) was then added. In a few seconds separation of sulphonamide began and the solution became moderately warm. Active chlorine rapidly disappeared from the solution and the odour of dichloroacetone became marked. The solution was neutralised with acetic acid and distilled, when oily drops of dichloroacetone collected in the distillate. It was identified by its reactions with phenylhydrazine, Fehling's and alkaline silver solutions. Another portion was warmed with excess of *p*-nitrophenylhydrazine in dilute sulphuric acid, when a 30 % yield of characteristic methylglyoxal-*bis*-*p*-nitrophenylhydrazone was obtained. The substance was dissolved in boiling pyridine and crystallised by adding an equal volume of absolute alcohol and was again crystallised from nitrobenzene and dried *in vacuo* at 150°. It melted at 300–302° and gave the following result on analysis:

0·1345 g.; 29·5 cc. moist N at 20° and 758 mm.

Found ... ... 24·9 % N.

Calculated for $C_{15}H_{14}O_4N_6$ 24·6 % N.

Oxidation of valine.

Valine (1·17 g.) was oxidised in neutral 1 % aqueous solution with chloramine-T (1 mol.). The reaction was rather slow and the mixture required warming at 37° for an hour and a half before active chlorine disappeared. The solution was then neutralised with acetic acid and the volatile isobutyric aldehyde distilled off into nitrophenylhydrazine sulphate solution. The characteristic orange yellow nitrophenylhydrazone (2·3 g.) was crystallised from alcohol and melted at 132–133°. This derivative has already been described [Dakin, 1908]. The substance was analysed with concordant results.

On carrying out the oxidation with two equivalents of chloramine-T, qualitative evidence of the formation of isopropyl cyanide was easily obtained but the yield was too small for satisfactory identification. The formation of this cyanide is analogous to the conversion of leucine into isobutyl cyanide previously referred to in the introduction.

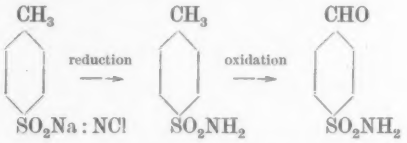
Oxidation of isoleucine.

Synthetic isoleucine (2·62 g.) was oxidised with chloramine-T (5·7 g.) as described in the case of valine. The reaction seemed to go smoothly and ammonia and carbon dioxide were promptly noticeable. The volatile methylethylacetaldehyde was rather difficult to characterise since most of

the hydrazine and other derivatives were oily or crystallised with difficulty. Nitrophenylhydrazine was found to give a crystalline derivative, but it was very soluble in most solvents and difficult to crystallise satisfactorily. The aldehyde was therefore oxidised to methylethylacetic acid with moist silver oxide. The silver salt on analysis was found to contain 51·8 % of silver compared with a calculated value of 51·6.

*Benzaldehyde-*p*-sulphonamide and its derivatives.*

On heating 5 % glucose solutions at water-bath temperature with one, two or three equivalents of chloramine-T, oxidation occurs rather slowly and toluenesulphonamide is precipitated. On treating the filtrate at room temperature with phenylhydrazine acetate, a voluminous yellow precipitate is obtained which at first was thought to be a sugar derivative. The substance on examination proved to be the hydrazone of benzaldehyde-*p*-sulphonamide. The yield of product was considerable, *e.g.* 3 g. glucose when oxidised with 9 g. chloramine-T gave 1·3 g. of pure hydrazone. Apparently the aldehyde is produced from *p*-toluenesulphonamide by oxidation with unchanged chloramine-T; at any rate, its production is noted when the two substances are heated together in aqueous solution.



Subsequently it was found that the aldehyde itself could be separated with ether by prolonged extraction of the aqueous filtrate referred to above. The ether solution on evaporation gave a mixture of the aldehyde with *p*-toluenesulphonamide. The pure aldehyde was most readily obtained by decomposing its "anil" derivative obtained by adding aniline, dissolved in alcohol, to an aqueous extract of the ether residue, or direct to the crude filtrate from the toluenesulphonamide.

p-*Sulphonamidobenzylideneaniline* $(SO_2NH_2) \cdot C_6H_4 \cdot CH : N \cdot C_6H_5$.

This substance, obtained as just described, by adding an alcoholic solution of aniline to an aqueous solution containing benzaldehyde-*p*-sulphonamide, is precipitated as a greyish white precipitate, which on crystallisation from boiling water is obtained as a felted mass of silky crystals melting sharply at 208°. It has the usual properties of anils and is readily decomposed by acids.

Analysis: 0·1168 g.; 0·2563 g. CO_2; 0·0470 g. H_2O;
0·1381 g.; 12·6 cc. moist N at 16° and 766 mm.

	C	H	N
Found %	59·9	4·5	10·7
Calculated for $C_{13}H_{12}O_2N_2S$ %	60·0	4·6	10·8

Benzaldehyde-p-*sulphonamide.* On warming *p*-sulphonamidobenzylideneaniline with ten parts of 10 % sulphuric acid for a few minutes on the water-bath, the aldehyde is readily set free. On repeated extraction with ether, the aldehyde is slowly extracted and is obtained as a crystalline residue on evaporation of the ether. The crude substance is recrystallised from boiling water, filtered from a trace of oily impurity, and is obtained in the form of shining colourless plates and needles melting at 122–124°.

Benzaldehyde-*p*-sulphonamide is readily soluble in warm water, moderately soluble in cold water. It is readily soluble in methyl or ethyl alcohol and acetone but much less soluble in ether. It is sparingly soluble in chloroform, insoluble in petroleum. The aldehyde appears to be quite stable when exposed to the air and undergoes oxidation with some difficulty.

Analysis: 0·1153 g.; 0·1915 g. CO_2; 0·0388 g. H_2O,
0·1000 g.; 6·8 cc. moist N at 17° and 764 mm.

	C	H	N
Found %	45·3	3·7	7·8
Calculated for $C_7H_7O_3NS$ %	45·4	3·8	7·6

Benzaldehyde-p-*sulphonamide hydrazone.* On adding hydrazine hydrochloride to an aqueous solution of benzaldehyde-*p*-sulphonamide a gelatinous precipitate of the hydrazone is obtained which becomes granular on gentle warming and standing. It is very sparingly soluble in boiling water but may be crystallised from 80 % alcohol in which it is moderately soluble. It forms flat shining plates and needles melting at 288–290°.

Analysis: 0·1029 g.; 0·1608 g. CO_2; 0·0405 g. H_2O.

	C	H
Found %	42·6	4·4
Calculated for $C_7H_9O_2N_3S$ %	42·2	4·5

Benzaldehyde-p-*sulphonamide phenylhydrazone.* The formation of this substance has already been described. It is obtained as greenish gold shining plates melting with decomposition at 244–245° when crystallised from hot alcohol in which it is sparingly soluble.

Analysis: 0·1733 g.; 0·3568 g. CO_2; 0·0755 g. H_2O.

	C	H
Found %	56·2	4·8
Calculated for $C_{13}H_{13}O_2N_3S$ %	56·7	4·7

Benzaldehyde-p-*sulphonamide semicarbazone.* This substance was obtained by adding excess of semicarbazide hydrochloride and sodium acetate to an aqueous solution of the aldehyde. The semicarbazone crystallises from water, in which it is sparingly soluble, in the form of characteristic twin prisms and melts at 250–251° with a little sintering a few degrees below. It is not readily soluble in either acid or alkali.

Analysis: 0·1801 g.; 0·2590 g. CO_2; 0·0665 g. H_2O.

	C	H
Found %	39·2	4·1
Calculated for $C_8H_{10}O_3N_4S$ %	39·6	4·1

REFERENCES.

Alefeld (1909). *Ber.* **42**, 1426.
Dakin (1908). *J. Biol. Chem.* C, **4**, 237.
—— (1916, 1). *Biochem. J.* **10**, 319.
—— (1916, 2). *Proc. Roy. Soc.* B, **89**, 232.
Grimaux and Adam (1880). *Bull. Soc. Chim.* [2] **34**, 29.
Harries and Alefeld (1909). *Ber.* **42**, 159.
Kling (1909). *Compt. rend.* **148**, 568.
Knorr (1888). *Ber.* **21**, 1205.
Langheld (1909). *Ber.* **42**, 2371.
Perkin (1899). *J. Chem. Soc.* **75**, 11.
Will (1891). *Ber.* **24**, 3831.
Zelinsky, Annenkoff and Kulikoff (1911). *Zeitsch. physiol. Chem.* **73**, 467.

XI. A RAPID PROCESS FOR THE ESTIMATION OF THE HIGHER FATTY ACIDS AND SOAPS IN FAECES.

By JOHN SMITH SHARPE.

(From the Department of Physiology, Glasgow University.)

(Received April 25th, 1917.)

In connection with an investigation on the aetiology of rickets at present being carried out under the Medical Research Committee, the necessity was experienced of a rapid and accurate method for the determination of fat in the faeces. That such a method would prove of value in clinical work is obvious, as it would facilitate the regulation of the diet of patients under treatment. It was therefore with this end in view that the present work was commenced. Various processes for the estimation of the fat content were tried. The results obtained with the staining method were far from accurate and this was rejected.

The acid amyl alcohol separation used for milk did not give a satisfactory result owing to the difficulty of obtaining an average of the faeces when made liquid with water, as sedimentation of the insoluble matter rapidly took place which carried down with it some of the fat.

On further consideration it was thought that the first step in the determination of the fat content should be the use of a suitable solvent. A 2 % alcoholic solution of caustic potash was employed since by this treatment all the fat is converted into the corresponding soap from which the cloud of fatty acid is displaced and determined by the nephelometer [Kober and Graves, 1915].

The only work which I can find directly bearing on the present investigation is by Laws and Bloor [1916] who extract the faeces with acidified alcohol and ether and seem to obtain fairly consistent results, which they claim to be within 5 % of those obtained by older methods.

By employing an acid medium for extraction it is however possible that there may be, on dilution, a recombination of the calcium and magnesium with some of the fatty acids, thus giving a low result.

Method.

(1) *Total Fatty Acids, i.e.* free and combined as neutral fats and soaps.

An average sample of the faeces is taken, thoroughly mixed and 1 g. weighed on to a fat-free filter paper, the material being spread out so as to form a thin layer. It is then introduced into a conical flask, 50 cc. of 2 % alcoholic potash solution are added and the flask is connected with a reflux condenser. Boiling is continued for about 15 minutes, by which time all the fat becomes saponified. The solution is cooled and decanted into a 200 cc. flask, made up to the mark, thoroughly mixed and a quantity filtered into a dry beaker. Of the filtrate 2 or 5 cc., depending on the quantity of fat present, are pipetted off and placed in a 100 cc. measure, 5 cc. of hydrochloric acid are added and the whole made up to 50 cc. with distilled water and mixed.

The standard is prepared as nearly as possible at the same time by taking 2 or 4 cc. of a standard fatty acid solution (1 cc. = 0·0005 g. fatty acids) made by saponifying oleic acid[1] 50 %, palmitic acid 25 % and stearic acid 25 % with alcoholic potassium hydroxide, and treating with hydrochloric acid in the same way as the sample. Both volumes are then made the same and the measures are set aside for about five minutes to allow the clouds of suspended fat to develop and the clouds are then compared in the nephelometer. The quantity of fat in the faeces can then be calculated. There is no colour in the sample tube when the cloud is precipitated, which makes the comparison of the fields very easy.

Results.

(1) *Test of the nephelometric process.* To ensure a good average for the test the faeces were dried, hence the high percentage of fatty acids. This was done by slow evaporation with alcohol on the steam-bath, care being taken not to overheat.

A. Sample from a child with rickets.

(1) Nephelometric method $\left\{\begin{array}{l}\text{reading of standard} \quad \ldots \quad \ldots \quad 60 \\ \text{,,} \quad \text{,, sample (average)} \quad \ldots \quad 68\end{array}\right\}$,

$$\therefore \frac{60 \times 0{\cdot}002}{68} = \text{quantity of fatty acids in amount of sample used}$$

$$= 17{\cdot}60\ \%\ \text{fatty acids.}$$

(2) By Soxhlet's extraction = 17·68 % ,, ,,

[1] It was found that oleic acid was necessary to produce the fat cloud. Dilute soap solutions of palmitic and stearic acids when acted on by HCl flocculated and settled as a curd, but on addition of the slightest trace of oleic acid the cloud so formed remained in complete suspension indefinitely.

A. Two days later.

(1) Nephelometric method { reading of standard ... ... 60 / „ „ sample (average) ... 81 } = 14·8 % fatty acids.

(2) By Soxhlet's extraction ... ... = 14·7 % „ „

B. Child with rickets.

(1) By nephelometer { reading of standard ... 60 / „ „ sample (average) 59 } = 20·3 % fatty acids.

(2) „ Soxhlet's extraction ... ... = 19·9 % „ „

(3) „ ethereal extraction after liberation of the fatty acids by HCl ... ... = 20·0 % „ „

B. Two days later.

(1) By nephelometer { reading of standard ... 60 / „ „ sample (average) 65 } = 18·4 % fatty acids.

(2) „ Soxhlet's extraction ... ... = 17·9 % „ „

(3) „ ethereal extraction after liberation of the fatty acids by HCl ... ... = 18·0 % „ „

It will be seen that the results are well within the limits of experimental error.

For accurate work in cloud reading the nephelometer is indispensable. Nesslerising tubes may however be substituted, and when used with care give a fair degree of accuracy. When using the comparison tubes all that is necessary is to produce the cloud in a similar quantity of the sample, as described above, and, using a second tube, run in from a burette the standard fatty acid solution, diluted if necessary, until the densities of the clouds are alike. From the number of cc. used the quantity of fatty acids in the faeces can readily be found.

Example:

(*a*) cc. of standard soap sol. used = 4·0; 20·0 % fatty acids.

(*b*) „ „ „ „ = 3·7: 18·7 % „ „

By Soxhlet's extraction ... ... 19·9 % „ „

(2) *Soaps.* (i) Sodium and potassium soaps (soluble soaps).

For the estimation of the soluble soaps it is only necessary to extract with distilled water and proceed exactly as above. Of course a larger quantity of the sample must be taken as there is a very small percentage of fat combined

in this form. The average amount in the samples I have examined is about 0·2 % calculated as fatty acid.

(ii) Calcium and magnesium soaps (insoluble soaps).

There is no direct nephelometric method for the estimation of these insoluble soaps in the faeces. It is necessary to know the ether-soluble fatty acids, as well as the total fatty acids and the water-soluble soaps.

Example:	Sample A	Sample B
Total fatty acids by nephelometer[1] ...	18·4 %	20·3 %
Fatty acids by Soxhlet	17·9 %	19·9 %
= Total soaps (by difference)	0·5 %	0·4 %
Water-sol. soaps by nephelometer ...	0·2 %	0·2 %
= Ca and Mg soaps (by difference) ...	0·3 %	0·2 %

With regard to the time necessary to complete a series of these estimations it was found that from eight to ten samples could be undertaken in a day. Thus the process offers a very great advantage over the tedious method of extraction with ether.

Another point in favour of the method is that all the fatty material is extracted whether combined as neutral fats, fatty acids or soaps and estimated as total fatty acids. This overcomes the possibility of incomplete ether extraction which is a well-known difficulty.

Conclusions.

By the saponification process and the use of the nephelometer very rapid and accurate determinations of the fatty acid content in faeces can be made. To ensure good results the following points require attention.

(*a*) The clouds in the sample and standard should be precipitated at the same time.

(*b*) The same amount of HCl should be added to each.

(*c*) The same time (about five minutes) should be allowed for the clouds to form.

(*d*) A standard should be used which gives readings fairly close to those of the sample analysed.

The application of the method to substances other than faeces is being investigated.

[1] I have evidence in work on fat metabolism in rickets, as yet unpublished, to show that there is very little neutral fat present in the faeces. The fatty acids are for the most part free, with a very small quantity combined as soaps (see above).

I have to thank Professor Noël Paton for the advice he gave me during this work. I am also indebted to Mr David Burns who provided the nephelometer from a grant by the Carnegie Trustees.

The other expenses connected with the work were defrayed by a grant from the Medical Research Committee to whom I desire to express my thanks.

REFERENCES.

Kober and Graves (1915). *J. Ind. Eng. Chem.* (American), **7**, 843.
Laws and Bloor (1916). *Amer. J. of Diseases of Children*, **11**, No. 3.

XII. THE DISTRIBUTION OF NITROGEN IN BEER.

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(*Received May 25th, 1917.*)

The value of beer as a food has been for long a subject of controversy. There can be no doubt that the alcohol and sugars have a certain value as sources of energy, but whether the nitrogenous compounds are of any value has never been fully investigated.

In the analysis of beer it is customary to determine the nitrogen content, to multiply this by 6·25 and to express the result as protein—a totally fallacious procedure.

In the Report of the Committee of the Royal Society on the Food Supply of the United Kingdom it is stated "that the nitrogen compounds of beer have been little studied," and so incomplete is our knowledge as regards their nature that the committee merely made a guess in assuming that something between 50 and 100 % of the nitrogen is in the form of protein or amino-acids which are utilisable in the body.

The object of the present paper is to throw further light upon the nature of these nitrogenous compounds.

Fate of the Proteins of Barley during the Brewing Process.

As a result of the malting, part of the protein in the grain is acted on by proteolytic enzymes which break it down into simpler substances. These along with a little soluble protein pass into solution during the mashing but the greater part is left in the brewers' grain. During the processes of boiling, hopping and subsequent cooling of the wort some of this protein becomes precipitated.

The amount of nitrogen thus finally left in solution is termed by the brewer "permanently soluble nitrogen." By the action of the yeast during fermentation a further quantity of soluble nitrogen is removed by these organisms using a certain proportion as food.

A further amount of protein is removed after the beer is made and this process is carried out to ensure that the beer may remain "bright."

The following table is taken from analyses by Bungener and Fries [quoted by Chapman, 1912, p. 14]. It shows the increase in the soluble nitrogen in the production of malt from barley.

TABLE I.

	Barley	Malt
Total nitrogen	1·690 %	1·580 %
Nitrogen as albumin	0·161	0·230
„ peptone	0·040	0·060
„ amides	0·206	0·534
Total soluble nitrogen	0·355	0·642

Thus it will be seen that in barley there is about 20 % of the total nitrogen in a soluble form, while in malt almost 40 % exists in this form. Of this 40 %, which during the period of mashing goes into solution, a certain proportion as already stated remains in the wort as "permanently soluble nitrogen." A large amount of research work on these bodies has been undertaken by Horace Brown [1907].

He accounts for almost 66 % of this "permanently soluble nitrogen" as the following table details:

TABLE II.

Ammonia nitrogen	3·5 %
Malt-albumose nitrogen	20·0
Malt-peptone nitrogen	31·0
Amide and amino-nitrogen	8·5
Nitrogen due to organic bases ...	4·0
Balance (unclassified)	33·0
	100·0

The 4 % of nitrogen due to organic bases comes from betaine and choline, and allantoin (glyoxyldiureide) was also present on the wort (Horace Brown).

The proportion of nitrogen capable of being assimilated by the yeast was ascertained by Horace Brown. By successive croppings of yeast and the estimation of the nitrogen content of the wort before and after fermentation he found that almost 60 % could be accounted for by the yeast action. This was a laboratory experiment and the action of the yeast was continued much longer than it is in practice. However the same test was carried out under brewing conditions with the result that the assimilated nitrogen varied between 20 and 36 % of the permanently soluble nitrogen.

From the foregoing it will readily be seen that the protein content of the beer or ale will not be appreciable when it reaches the consumer.

Application of Protein Tests.

1. The biuret test was tried both directly and on the isolated protein bodies. In every case negative results were obtained. The same result has also been obtained by Horace Brown.

2. Millon's reagent gave a negative result.

3. The ninhydrin test was also negative.

4. The xanthoproteic test alone gave a positive result.

Table III gives the percentage of so-called protein in different ales, calculated from the total nitrogen as given in the second column [Chapman 1912].

TABLE III.

	Proteins	Total nitrogen
London Mild Ale	0·33 %	0·052 %
Burton Pale Ale	0·50	0·080
English Strong Ale	0·91	0·145
Dublin Stout	0·75	0·120
American Ale	0·57	0·091

The only figures for the distribution of nitrogen in beer which I have been able to find are by Voltz, Förster and Baudrexel [1910] who give an analysis of North German dark beer used by them in their investigation of the absorption and general action of beer.

TABLE IV.

Alcohol	4 %
Extract	5·7

Analysis of Extract.

Ash	3·44
Organic	96·56
Nitrogen × 6·25	6·36 = 1·01 % N.
Ethereal extract	0·13
Nitrogen-containing substances:	
Protein	42·7
Amide-N.	57·3

METHODS.

(1) Total Nitrogen. This was determined by the Kjeldahl method using 10 cc. of the sample. The Kjeldahl-Jodlbauer [see Sutton, 1904, p. 87] modification was tried but no advantage was gained since no nitrogen seems to exist as nitrates or nitrites.

(2) Protein Nitrogen. 25 cc. of the sample were evaporated to half bulk and then cooled to about 10°, 10 % tannic acid solution containing 2 % phosphoric acid (about 4 vols. to 1 of sample) was added, shaking the while,

until no further precipitate formed. The protein mass was allowed to flocculate, then filtered off on nitrogen-free filter paper and washed with the precipitating solution. The nitrogen in the precipitate was then determined by the Kjeldahl method.

Nitrogen found × 6·25 = Total Protein.

Tannic acid solution used by itself as a precipitant, may bring down bases along with the protein but this is prevented by the solvent action of the phosphoric acid. By too lavish use of tannic acid an error may be incurred, (it is a case of the mutual precipitation of two colloids) with consequent loss of protein [Barger 1914, p. 117].

Other methods of taking out the proteins were tried but it was found that the above gave the most satisfactory results. The lead acetate precipitation was investigated. This salt has a well recognised power of throwing proteins out of solution, especially if a little of the tribasic acetate is added. The results with this were far from satisfactory as the following figures will show:

Protein nitrogen by tannic acid method	0·021 %
,, ,, acetate method	0·011 %

The failure of lead acetate to precipitate all the protein is difficult to explain, unless it be that the metallic salts of these plant proteins are more soluble than those in the corresponding animal series. Further light on this point would be interesting.

The use of (*a*) copper acetate or (*b*) phosphotungstic acid as a precipitating agent is open to serious objection as both precipitate purine and basic bodies. These were therefore rejected.

(3) Amino-acid Nitrogen. The estimation of the nitrogen combined in this form was effected by the nitrous acid method of van Slyke [1912]. 5 cc. of the ale or beer were run into the deaminising flask and this was acted on by 20 cc. of the nitrous acid mixture. Numerous duplicate estimations were performed and an average of the whole was taken. Amide-nitrogen is included in the figures given.

A full description of the process employed by previous workers for the separation of the amino-acid and amide-nitrogen is given by Horace Brown [1903].

(4) Purine Nitrogen. (*a*) *Qualitative Tests.* The presence of purine bodies was proved by the following tests:

1. The murexide test.
2. Precipitation by ammoniacal silver nitrate and magnesia mixture.
3. Precipitation after adding copper sulphate and sodium bisulphite.

4. The intense blue colour produced on adding phosphotungstic acid reagent (Folin) to the material made alkaline with sodium carbonate. This Folin considers characteristic of uric acid (trioxypurine).

(*b*) *Quantitative Tests.* The estimation of the uric acid content was carried out by the new method of Folin and Denis [1913] for its estimation in urine. It involves the precipitation as a silver magnesium compound with subsequent treatment by the phosphotungstic acid reagent and comparison of the colour produced with a standard. This method was used in the case of dark coloured beer and stout where the original colour of the material would have interfered greatly with the delicacy of the reaction. It was however found that in light ales or beers quite accurate results were obtained by direct treatment with Folin's reagent, but that care had to be exercised not to allow too much time to elapse after the addition of the reagent owing to the liability of the colour to fade on standing longer than five minutes. Of the beer under examination 2 cc. were therefore drawn off and placed in a test tube; 5 cc. of saturated sodium carbonate solution were next added and then from ten to twenty drops of the phosphotungstic acid. The mixture was shaken together and slightly heated to facilitate the reaction. This was washed into a Nesslerising-tube to about 25 cc.

The standard was prepared in a similar tube by mixing together 20 cc. of water, 5 cc. of saturated sodium carbonate solution and about 20 drops of the phosphotungstic acid, a lithium carbonate solution of uric acid (1 cc. = 1 mg. uric acid) was then delivered from a burette into the tube containing the standard until the depth of colour in each was alike. Both volumes were then made the same. These were further compared in a Duboscq colorimeter and any necessary adjustment made. The number of cc. of the standard solution used multiplied by 0·333 gives the equivalent in trioxypurine nitrogen[1].

(*c*) *Xanthine-like purines.* These were determined by a modification of Salkowski's [1895] method for urine. The proteins were removed by lead acetate, the lead removed by H_2S gas. The phosphates were removed from the filtrate by magnesia mixture. Ammoniacal silver nitrate was then added and the resulting precipitate was filtered off and suspended in water, decomposed with H_2S and filtered. The filtrate was evaporated to a syrup and the residue treated with a little water containing 2 % of sulphuric acid which dissolves the xanthine bases leaving the uric acid practically insoluble.

The purine bases were reprecipitated by ammoniacal silver nitrate and

[1] A table giving the purine content of some beers and ales is given by C. von Noorden [1907].

either estimated by the quantity of silver in the precipitate or by the quantity of nitrogen given by the Kjeldahl method. For this work the nitrogen in the precipitate was estimated.

Xanthine seems to form the greater bulk of these more soluble purines. Confirmatory tests were given by the base liberated from the silver compound. The following may be quoted:

(*a*) Strecker's test. On evaporating the residue on a watch glass with a drop of nitric acid a yellow residue remains which turns reddish yellow on addition of potassium or sodium hydroxide.

If ammonia be substituted for the fixed alkali, no violet coloration is obtained. This is a distinction from uric acid.

(*b*) The residue was soluble in nitric acid and on crystallising, characteristic nitrate crystals were formed.

(*c*) Solutions of the residue gave a yellow crystalline precipitate with sodium picrate; at first it seemed to be amorphous, then assumed the form of round bunches of crystals.

Hypoxanthine, adenine and guanine were tested for, but none of these was found. Methylpurines were in every case absent.

The proportion of nitrogen existing in the different forms of combination found by the above processes, *b* and *c*, varies greatly. Thus the nitrogen as trioxypurine may be as much as 90 % of the total purine nitrogen or as low as 15 or 20 %, the sum being completed by the nitrogen combined in the xanthine-like bodies.

The "purine nitrogen" reported in Tables V to VIII is the sum of the nitrogen found by the above two processes *b* and *c* (p 105). This gives the total purine nitrogen.

(5) Total solid matter. This was estimated by drawing off 10 cc. from a good average sample of the beer or ale in question. It was then placed in a tared evaporating basin and evaporation continued on a water bath until dry. It was then transferred to a vacuum desiccator and dried until constant in weight.

(6) Carbohydrate. The amount of carbohydrate was determined after precipitation of the protein using Wiley's mercuric nitrate method [see Allen 1914, p. 157]. 25 cc. of the material were placed in a 100 cc. flask, 10 cc. of mercuric nitrate solution were added and the whole thoroughly mixed. When the froth settled, water was run in up to the mark on the neck and the contents again shaken. No allowance need be made for the precipitate in this case as it is so very small. When the supernatant liquid

was clear, a little (about 30 cc.) was filtered off through a fine paper (Green's 602). The concentration of sugars was then found by the polarimeter the whole being calculated to dextrin.

The results obtained by calculating the rotation all to dextrin are slightly low, about 0·3 % where a 3 % dextrin content is given. This is due to the maltose present having a slightly less specific rotation than dextrin.

The formation of an osazone derivative was tested for. Maltose was proved by the formation of maltosazone crystals, but no glucosazone was found.

Levulose was tested for, but negative results were obtained (Selivanov's resorcinol test). This sugar is very easily fermented by yeast whereas dextrin is not acted on, hence the latter is, on an average, a little above 66 % of the total carbohydrate present, the remainder being maltose [Chapman 1912].

Results.

The following tables show the results of analyses of some typical beers, ales and stouts. The volume of each sample is noted.

TABLE V.

Beer (light).

	Tennent's Lager Beer		Draught Beer	
Total nitrogen	—	0·040 %	—	0·57 %
Protein nitrogen	0·006 %	—	0·021 %	—
=Total protein	—	0·038	—	0·133
Amino-acid nitrogen	0·017	—	0·014	—
Purine nitrogen	0·016	—	0·019	—
Undetermined nitrogen	0·001	—	0·003	—
Total solid matter	4·89 %		3·47 %	
Carbohydrate (dextrin)	2·46		1·30	
Specific gravity	1006·8		1006·1	
Bottle contained	250 cc.		550 cc.	

TABLE VI.

Beer (strong).

	Bass & Co.'s Pale Ale (A)		Bass & Co.'s Pale Ale (B)	
Total nitrogen	—	0·084 %	—	0·082 %
Protein nitrogen	0·011	—	0·016	—
=Total protein	—	0·074	—	0·102
Amino-acid nitrogen	0·036	—	0·040	—
Purine nitrogen	0·039	—	not done	
Undetermined nitrogen	0·004	—		
Total solid matter	6·37 %		6·20 %	
Carbohydrate (dextrin)	3·06		3·00	
Specific gravity	1009·4		1009·0	
Bottle contained	330 cc.		265 cc.	

TABLE VII.

Ales.

	Younger's Edinburgh Pale Ale		Worthington's Indian Pale Ale	
Total nitrogen	—	0·039 %	—	0·063 %
Protein nitrgoen	0·010 %	—	0·010 %	—
=Total protein	—	0·063	—	0·063
Amino-acid nitrogen	0·018	—	0·016	—
Purine Nitrogen	0·010	—	0·033	—
Undetermined nitrogen	0·001	—	0·004	—
Total solid matter	4·89 %		4·92 %	
Carbohydrate (dextrin)	2·46		2·24	
Specific gravity	1008·3		1007·0	
Bottle contained	535 cc.		330 cc.	

TABLE VIII.

Porter.

	Allsopp's Special Stout		Guinness's Extra Stout	
Total nitrogen	—	0·056 %	—	0·113 %
Protein nitrogen	0·012 %	—	0·029 %	—
=Total Protein	—	0·080	—	0·185
Amino-acid nitrogen	0·019	—	not done	
Purine nitrogen	0·020	—		
Undetermined nitrogen	0·005	—		
Total solid matter	5·90 %		—	
Carbohydrate (dextrin)	2·05		—	
Specific gravity	1009·9		1010·0	
Bottle contained	305 cc.		310 cc.	

(All the above analyses were made in duplicate.)

Table IX shows the percentage on the total nitrogen of the nitrogen combined in various forms.

TABLE IX.

	Tennent's	Draught	Bass's	Younger's	Worthington's	Allsopp's	Guinness's
Protein N in total N	15·1	37·0	13·2	25·8	15·9	21·4	25·0
Amino N ,,	42·5	24·5	35·7	46·0	25·4	33·8	—
Purine N ,,	40·0	33·2	46·4	25·8	52·4	35·7	—
Undetermined N in total N	2·4	5·3	4·7	2·4	6·3	9·1	—
	100	100	100	100	100	100	—

Consideration of results.

(1) From a study of Tables V to VIII it will be seen how close the figures for the total nitrogen are, to those given in Table III from which previous workers have calculated the total protein. According to the present investigation only about 22 % of the total nitrogen exists as protein.

(2) Horace Brown [1907] in his researches on malt extract obtained a mixture of crystalline substances. These proved to be asparagine (amino-succinamide), tyrosine (*p*-hydroxyphenyl-*α*-aminopropionic acid) and leucine (isobutyl-*α*-aminoacetic acid).

These amino-acids will ultimately find their way into the beer and it is probable that they may be the source of the amino-nitrogen.

(3) As regards the purines there is some evidence to show that these bases may be derived directly from the malt, hops, and yeast. A. C. Chapman has isolated adenine, hypoxanthine and xanthine from these but he does not give data as to the percentage of the bases.

With regard to the malt and yeast the presence of purine bases in these seems to be fairly well established. As far as the present investigation is concerned xanthine (dioxypurine) and uric acid (trioxypurine) were present in comparatively large quantities. Further, since these have been found in the present work and allantoin has been found in wort by previous workers [Brown 1907] it is possible that an addition to the purine nitrogen may result from the hydrolysis and oxidation of nucleic acid [Mathew, 1915, p. 729], partly from the grain and partly from the yeast.

Examination of the Undetermined Nitrogen.

(1) *Alkaloids.*

On extracting a quantity of the beer made alkaline by sodium hydroxide with chloroform and evaporating off the solvent, a clear oil was obtained which turned brown on exposure to air, and assumed an odour very like that of tobacco juice from a pipe. It seemed also to be very volatile as it had to be dried in a desiccator for fear of loss which rapidly took place on drying at 100°.

A. Colour tests on the isolated alkaloid.

(i) A pale red colour was immediately produced by a drop of concentrated sulphuric acid in the cold. This colour was discharged on addition of a trace of nitric acid.

(ii) Sulphomolybdic acid gave a blood red colour in the cold which became darker on heating.

(iii) On evaporation with nitric acid a yellow colour was produced which turned rose pink on addition of alcoholic potash.

(iv) A deep pink colour was produced on heating with concentrated hydrochloric acid. This colour is discharged on addition of alkali.

B. The alkaloid dissolved in hydrochloric acid and in nitric acid quite freely and was liberated on addition of excess of caustic soda giving off the same odour as it had when extracted. It was proved by experiment that this alkaloid was not precipitated from its solutions by the tannic acid mixture used for the protein extraction, it is therefore another source of nitrogen.

The alkaloid seems to belong to the pyridine group. Many of the reactions are typical of "coniine" which has been found in hops by Chapman [1914]. The following table shows the percentage of alkaloid in a few samples of beer etc. examined.

TABLE X.

	Alkaloid
Draught Beer, Paterson & Co. ...	0·007 %
Younger's Edinburgh Ale	0·004
Allsopp's Stout	0·010
Guinness's Stout	0·012

(2) *Bases.*

These were taken out with a solution of phosphotungstic acid. The precipitate was filtered off and suspended in water, decomposed with barium hydroxide and filtered. The barium was removed by a current of CO_2 gas and the precipitate filtered off. Purines were then precipitated by ammoniacal silver nitrate solution, allowed to settle and filtered. The excess of silver was removed from the filtrate by hydrochloric acid and the solution concentrated to a syrup. Alcoholic mercuric chloride solution was added which precipitated the bases. This precipitate was decomposed with H_2S gas and the filtrate again concentrated. Picric acid solution was added and the whole set aside to crystallise. Bunches of yellow needles were formed which were fairly insoluble in water and had a melting point a little over 181°. This corresponds to the commonly occurring plant base, betaine, which has been found by previous workers in wort, see page 102. There was however an extremely small percentage of this in beer, only 0·005 % of the picrate, on an average, being isolated.

Conclusions.

1. The protein nitrogen of the beers analysed, varies from 13·2 to 37·0 % of the total nitrogen, *i.e.* the protein content of beer varies from 0·038 to 0·185 %.

2. The nitrogen as amino-acid varies from 25 to 46 % of the total nitrogen, *i.e.* the amino-acid nitrogen of beer varies from 0·014 to 0·040 %.

3. There is a varying proportion of nitrogen combined as purine compounds which in some cases exceeds that combined as amino-acid nitrogen. It varies from 25·8 to 52·4 % of the total nitrogen or from 0·010 to 0·039 % of the beer itself.

4. The undetermined nitrogen of these beers varies from 2·4 to 6·3 % of the total nitrogen, *i.e.* the percentage on the beer itself varies from 0·001 to 0·005.

5. The percentage of undetermined nitrogen is not quite made up by the addition of the nitrogen combined as alkaloid and base.

In the case of Allsopp's stout there is only 0·0011 % of nitrogen combined as the former and the stouts seem to contain the largest amount of alkaloid.

Of basic nitrogen there is only 0·0002 %, which is negligible.

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REFERENCES.

Allen (1914). *Commercial Organic Analysis*, **8**.
Barger (1914). *The Simpler Natural Bases* (Longmans, London).
Brown, Horace (1903). *Trans. Guinness Research Laboratory*, I. i. 18.
Brown, Horace (1907). *J. Inst. Brewing*, New Series, **4**, 394.
Chapman, A. C. (1912). *Brewing* (Cambridge, University Press).
Chapman, A. C. (1914). *J. Chem. Soc.* **105**, 1895.
Folin and Denis (1913). *J. Biol. Chem.* **14**, 95.
Mathew (1915). *Physiological Chemistry*.
Noorden, C. van (1907). *The pathology of metabolism*, **3**, 1297.
Salkowski (1895). *J. Chem. Soc.* **2**, 538.
Sutton (1904). *Volumetric Analysis*, 9th Ed.
Slyke, D. D. van (1912). *J. Biol. Chem.* **12**, 278.
Völtz, Förster and Baudrexel (1910). *Pflüg. Arch.* **134**, 133.

XIII. THE VISCOSITY OF SOLUTIONS.

By SVANTE ARRHENIUS.

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1. Theory of Einstein, p. 112. 2. Experiments of Bancelin, p. 113. 3. Experiments of Mifka, p. 113. 4. Woudstra's determinations, p. 114. 5. The logarithmic formula, p. 114. 6. Suspensions of sulphur, examined by Odén, p. 116. 7. Influence of hydratation, p. 116. 8. Influence of temperature on the value of $\theta = d \log \eta / dc$ for dilute solutions, p. 118. 9. Associating substances, p. 119. 10. Solutions of nitrocellulose, p. 120. 11. Solutions of rubber, p. 122. 12. Hatschek's formula for solvatation (hydratation), p. 124. 13. Viscosity of rice-starch suspensions, p. 125. 14. Irregularities, p. 127. 15. The values of k in formula (1 *a*), p. 129. 16. Summary, p. 131. References, p. 133.

1. *Theory of Einstein.* In recent years a great number of investigations, of theoretical or of experimental scope, has been carried out regarding the viscosity of solutions by different scientists, so that it seems possible to arrive at more definite ideas on this important question.

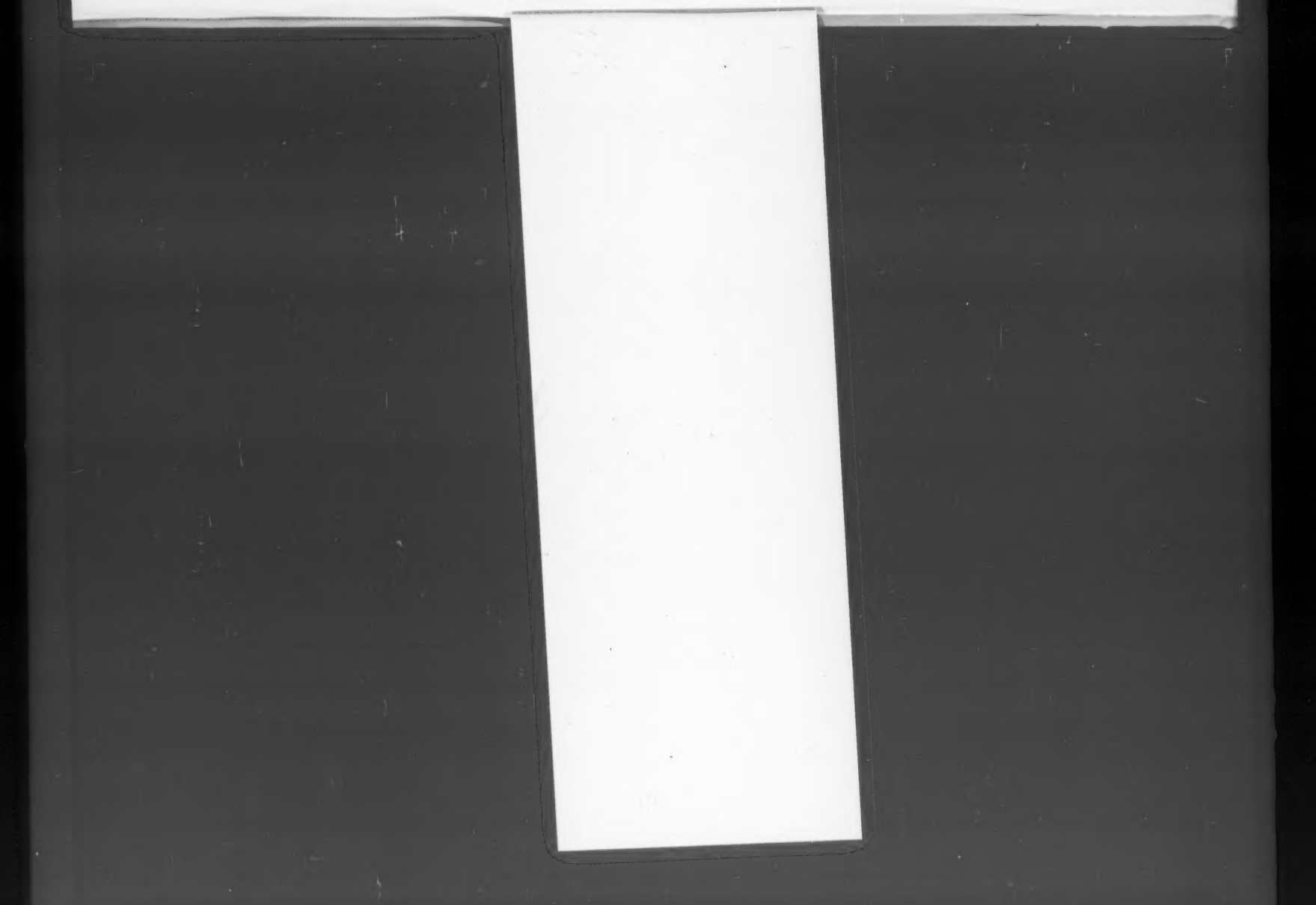
The chief theoretical investigations in this line are due to A. Einstein [1906, 1911] and von Smoluchowski [1916]. Einstein deduced in 1906 and 1911 the following formula:

$$\eta = \eta_0 (1 + 2{\cdot}5\, \phi) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

in which η is the viscosity of a solution containing the volume fraction ϕ of a dissolved substance in unit volume of a solvent with the viscosity η_0 at the same temperature. This formula is only valid for very dilute solutions for which $\sqrt[3]{\phi}$ may be neglected in comparison with 1. Further it is supposed that the dissolved particles are of spherical form and of great magnitude compared with the molecules of the solvent. The formula is deduced for the case of suspensions—*e.g.* so-called colloidal solutions of sulphur or of gamboge—but as there is a continuous transition from suspensions to genuine solutions, Einstein did not hesitate to apply his formula to solutions of cane sugar in water although the sugar molecules are probably not of spherical form.

ERRATA

page 115 line 16 from bottom, *for* Equér *read* Egnér.
118 last line, *for* solvent *read* solute.
120 line 7 from top, *for* disaggregation *read* aggregation.
128 line 9 from top, *for* 5·00 *read* 500.
130 line 9 from top, *for* great *read* greater.
133 line 11 from top, *for* Equér *read* Egnér.



2. *Experiments of Bancelin.* The said formula has been examined experimentally on suspensions of gamboge by Bancelin [1911, 1, 2] in the laboratory of Perrin. He found a good agreement with the theory, as the coefficient of ϕ was found equal to 2·7 (Bancelin gives 2·9, which is probably a misprint) whereas the theory demands 2·5. Still better is the agreement if we use the formula (2) below, as is seen from the following table:

% (volume)		Viscosity . 10^5		
measured	corrected	obs.	calc. 1	calc. 2
0	0	1016	1016	1016
0·09	0·09	1018	1018	1018
0·24	0·241	1023	1022	1022
0·33	0·331	1025	1025	1024
0·53	0·533	1029	1030	1029
0·66	0·665	1033	1033	1033
1·05	1·061	1044	1044	1043
2·11	2·155	1074	1074	1072

The measured percentage is given by Bancelin, the corrected one gives the volume of the gamboge granules, when the volume of the water is put equal to 100. This corrected value is, as easily seen, proportional to the molecular percentage of the gamboge. In the logarithmic formula (2) the concentration is determined in this manner (cf. § 5). The calculated values below calc. 1 are found if we suppose the constant θ in formula (2) to be 0·0111, corresponding to a value 2·56 of the constant in formula (1), whereas the figures below calc. 2 correspond to $\theta = 0{\cdot}01086$ or 2·5 in Einstein's formula. 2·56 corresponds to an empirical determination of the said constant. The agreement may be regarded as perfect in both cases.

Bancelin has made experiments with granules of different diameters, namely 0·3, 1·2 or 4 μ, and he always found the same value of the viscosity for the same percentage, which is also demanded by the theory of Einstein. Further this theory demands that the coefficient 2·5 shall be independent of the viscosity, η_0, of the solvent. Bancelin determined the viscosity of suspensions of gamboge in an aqueous solution of glycerol with a viscosity fifteen times that of water. His results were wholly concordant with the theoretical predictions. Experiments with methylene blue gave consistent results.

3. *Experiments of Mifka.* Some experiments of Mifka [1911] on suspensions of silver, gold and copper, prepared in water according to the method of Bredig, indicate that these suspensions possess the same viscosity as the solvent at the same temperature. The concentrations of these solutions were only 4·25, 3·48, 2·79 resp. of the thousandth part of 1 % (volume), so

that their viscosities ought not to differ more than about 0·01 % from that of the solvent according to Einstein's theory. This difference falls wholly within the errors of observation (here about 0·05 %), so that it may be maintained that these experiments are in accordance with Einstein's theory. The conclusion, which is sometimes given, that metallic suspensions in general possess the same viscosity as the solvent, would of course not be warranted, if it were possible to prepare much stronger solutions than those investigated by Mifka.

4. *Woudstra's determinations.* Some experiments of Woudstra [1908] on suspensions of silver in water, containing *in maximo* 0·49 g. per litre, which corresponds to 0·00464 % by volume, are said to have given viscosities that *in maximo* exceeded the viscosity of water by 4·57 %, whereas the theory of Einstein demands only 0·012 %. Woudstra's experiments were carried out by means of colloidal silver prepared according to Muthmann's method and dialysed during three weeks. His values are the following. c is the concentration in mg. silver per litre, ϕ the corresponding value in volume %—the specific gravity of silver taken as 10·53—η the viscosity, $\eta_{calc.}$ found from formula (2).

c	$\phi \cdot 10^5$	η	$\eta_{calc.}$
490	464	1·0457	1·0072
385	365	1·0098	1·0056
337	319	1·0057	1·0049
289	273	1·0045	1·0041
190	180	1·0021	1·0028
93	88	1·0013	1·0014

The values are calculated on the supposition that the relative viscosity of a solution containing 0·01 vol. % is 1·0154, whereas Einstein's formula gives 1·00025, *i.e.* a sixty times smaller value of the difference from η_0 (put = 1). We shall find later, that such great deviations occur—they are probably due to a highly flocculated state of the suspended particles, similar to that of aluminium hydroxide, precipitated from a dissolved aluminium salt by means of an alkali. The most concentrated solutions give much higher viscosities than those calculated. This discrepancy vanishes with time. Thus the viscosity of the most concentrated solution was diminished after standing thirty-seven days to 1·0077, or nearly the calculated value. An addition of salts also diminishes the viscosity of the solution.

5. *The logarithmic formula.* We have seen above that Bancelin found that the viscosity of a gamboge suspension in glycerol may be written $\eta_1\eta_2$ if η_1 is the viscosity of the glycerol solution without gamboge and if η_2 is the

viscosity of a suspension of the same volume percentage of gamboge in pure water, when the viscosity of water at the same temperature is put equal to unity. This result, which is in accord with Einstein's theory, naturally suggests the idea that if two aqueous solutions of two substances possess the same relative viscosity η, then the viscosity of an aqueous solution containing both substances in the same volume ought to be η^2. Now the two substances may be identical. If we then carry our deductions further we find that the viscosity η of an aqueous solution of the concentration c ought to be subject to the condition

$$\log \eta = \theta c \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(2)$$

where θ is a constant and the viscosity of water at the same temperature is put equal to unity. This is the logarithmic formula found by myself empirically in 1887 [Arrhenius, 1887]. For dilute solutions, which I investigated at that time, it is indifferent whether the concentration is determined in per cent. of mass, per cent. of volume or molecular per cent.—in reality I used volume per cent. But when greater concentrations are examined, it is, as Kendall [1913] showed, better to express c in molecular percentage. It is remarkable that a similar assertion is true regarding other properties of solutions, *e.g.* osmotic pressure. In fact Kendall proved that the logarithmic formula is very nearly followed by mixtures in all proportions of liquids, which do not chemically react upon each other, if the concentration is expressed in molecular percentage. Equér [1917] has recently made a series of determinations on mixtures of two fluids and corroborated the conclusion of Kendall.

This statement is of great importance for controlling Einstein's formula (1). It is only valid for extremely dilute solutions and, as Smoluchowski states "it would demand an enormous work of calculation to carry the approximation further according to the method of Einstein." I have therefore used the logarithmic formula for calculating the experiments of Bancelin and in this manner I found the value 2·56 for the coefficient in formula (1) instead of the value 2·7 calculated by Bancelin, whereas Einstein's theory demands 2·5. In this case the size of the "molecules" of the gamboge is so enormous that their number in a suspension of 2·11 vol. % may be neglected in comparison with the number of the molecules of the solvent water. The molecular percentage may therefore without any sensible error be assumed to be proportional to the quantity of gamboge divided by the quantity of water in the solution examined. The same is true for all suspensions or emulsions

or solutions of albuminous substances or, in general, for what are called colloidal solutions.

6. *Suspensions of sulphur, examined by Odén* [1913]. In general it has not been possible to prepare suspensions of higher concentration, except for sulphur, and it is of great value for us that Odén has determined the viscosities of such suspensions, which contained up to 50 g. sulphur in 100 cc. I have shown [1916] that these suspensions are subject to the logarithmic formula (2) within the limits of the errors of observation.

The formula of Einstein is not applicable to the calculation of these concentrated solutions. Therefore Smoluchowski tried to construct an empirical formula with two new arbitrary constants. This formula has no greater interest; it may suffice to give, under $\eta_{\text{Smo.}}$, the tabulated values calculated by means of this formula in order to represent the observed values, $\eta_{\text{obs.}}$, for submicroscopical suspensions of sulphur (diameter of particles about $0{\cdot}1\,\mu$). These observed values are the mean values for three temperatures, 5°, 20° and 40°. Below $\eta_{\text{Ein.}}$ are written figures calculated by means of formula (1) where the specific gravity of the sulphur is taken equal to 1·95; below $\eta_{\text{Arrh.}}$ figures founded on formula (2). c gives the weight (grams) of sulphur in 100 cc. of the suspension, c_1 the corresponding weight in 100 cc. of water. c_1 is proportional to the molecular percentage of the sulphur. k is the calculated constant in formula (1), which ought to be 2·5 in extreme attenuation. The viscosity of water is put equal to 1.

c	c_1	$100\,\phi$	k	$\eta_{\text{obs.}}$	$\eta_{\text{Ein.}}$	$\eta_{\text{Smo.}}$	$\eta_{\text{Arrh.}}$
5	5·13	2·63	3·84	1·101	1·066	1·079	1·103
12·51	13·38	6·58	4·35	1·286	1·164	1·238	1·290
25·02	28·76	13·16	5·38	1·708	1·329	1·656	1·730
50·03	67·72	26·33	10·25	3·700	1·657	4·000	3·635

The deviation of k from Einstein's value 2·5 as well as a comparison of $\eta_{\text{Ein.}}$ with $\eta_{\text{obs.}}$ indicate how far the figures of Odén differ from the formula (1). Smoluchowski's formula gives in reality the limiting value 2·5, but its agreement with the observed values is not very satisfactory, the mean deviation reaching 4·2 %. On the other hand the figures in the last column, calculated by means of formula (2) with only one arbitrary constant give an agreement within the errors of observation, the mean difference being only 0·9 %. We may conclude from this that the limiting value of k for $c = 0$ is 3·72, *i.e.* 1·5 times greater than Einstein's theory demands.

7. *Influence of hydratation.* In the calculation of Odén's figures by means of formula (2) it has been supposed that no water is chemically bound to the

spherules of sulphur; in reality we have no indication at all that sulphur enters into compounds with water, all chemical evidence is against such a supposition. Therefore the good concordance between formula (2) and the experimental results is of a very high value in this case. This concordance is still better for the observations regarding amicroscopical sulphur (diameter of the particles about 0·01 μ) in which case the mean difference is only 0·5 % for seven observations in the same concentration interval as above, when η changes in the proportion 1 to 5.

In other cases there is high probability that a certain quantity of water becomes bound to the dissolved substance, *e.g.* if we investigate albuminous substances in aqueous solution. In such cases the molecular percentage has a higher value than if no water were bound, and the increase of this quantity is proportionally greater the higher the concentrations treated, if we suppose that each gram of the protein binds the same quantity of water at all concentrations. This hypothesis is true for very dilute solutions, *i.e.* solutions which contain very few gram-molecules per litre. As the proteins examined have not been more concentrated than about 30 % and their molecular weight is of the order of magnitude of about 10,000, the highest concentrations have not been more than about 0·03 normal. We may therefore suppose that their tendency to bind water is nearly constant and make our calculations on that ground.

In such cases we have to determine two arbitrary constants in the formula (2) namely not only the constant θ but also the quantity n of water bound to one gram of protein substance for the calculation of the molecular concentration. I have carried out some such calculations for some proteins, examined by Miss H. Chick [Chick 1914; Chick and Lubrzynska 1914], and furthermore determined the specific gravity of these substances from that of their aqueous solutions and calculated the value of k in formula (1). The results are given in the following table:

Substance	Sp. gravity	θ	n	k
Egg albumin	1·36	0·0184	0·7	2·95
Serum albumin	1·375	0·0207	1·5	2·14
Euglobulin	1·372	0·0625	2·2	4·91
Pseudoglobulin	1·402	0·0402	1·2	4·83
Sodium caseinogenate	1·414	0·1127	1·0	15·1

Hatschek, who [1916, 1] has tried to discredit the value of formula (2) in this journal, has still conceded that it, "when applied to Miss Chick's viscosity figures, fits with remarkable accuracy, and the values of θ and n are constants over the whole range with slight exceptions."

8. *Influence of temperature on the value of* $\theta = d \log \eta / dc$ *for dilute solutions.* In order to show how associated substances, which form aggregates together with the solvent, behave when the temperature changes, I have calculated some figures of Noack [1886] regarding the relative viscosity of two aqueous solutions of ethyl alcohol containing 3·38 mol. % (= 8·21 weight %) of alcohol or 27·17 mol. % (= 12·55 weight %) of water and of two solutions of acetic acid containing 4·96 mol. % (= 14·82 weight %) of CH_3COOH and 4·77 mol. % (= 1·78 weight %) of water. As units of η for the strong solutions are taken the values for the most concentrated solutions, measured by Noack, viz. solutions containing 99·28 mol. % (= 99·72 weight %) of alcohol and 99·34 mol. % (= 99·8 weight %) of acetic acid. For the weak solutions the unit viscosity is that of water at the temperature indicated in the table.

	Ethyl alcohol		Acetic acid	
Temp. °C.	3·38 mol. %	82·83 mol. %	4·96 mol. %	95·23 mol. %
0	1·582	1·632	1·374	1·380
10	1·489	1·506	1·350	1·213
20	1·410	1·411	1·327	1·138
30	1·343	1·344	1·304	1·108
40	1·284	1·288	1·279	1·106
50	1·234	1·248	1·262	1·120
60	1·191	1·216	1·234	1·146

For the alcoholic solutions the relative viscosities decrease with increasing temperature. This decrease is more pronounced at lower temperatures than at higher ones. In the interval 0°–60° the total decrease of θ is about in the proportion 2·5 : 1. The decrease is much less for the acetic acid solution of 4·96 mol. % so that θ sinks only in the proportion of about 1·5 : 1 and for the solution of 4·77 mol. % water in acetic acid we find a minimum of η and thereafter, between 40° and 60° an increase with temperature, which seems to indicate that here no sensible thermal dissociation takes place. The viscosity measurements show a very pronounced maximum at a concentration of 77 weight % at all temperatures between 0° and 60°. It is probable that this maximum belongs to a definite compound, namely the monohydrate of acetic acid, $CH_3COOH + H_2O$, which corresponds to 76·93 weight %. The acetic acid of 95·23 mol. % (= 98·52 weight %) may therefore be regarded as a solution of this monohydrate in acetic acid. At lower temperatures some loose aggregates of the solvent (acetic acid) exist, which cause the diminution of θ with increasing temperature as in the other three cases treated above, but at higher temperatures these loose aggregates have nearly wholly disappeared, so that the solvent has a constant composition.

The alcoholic solutions behave in a quite different manner. The maximum viscosity belongs at 0° to a solution of 36 weight % (= 18·05 mol. %) and at 60° to a solution of 51 weight % (= 28·94 mol. %). At intermediate temperatures there is a continuous change between these two values—perhaps at higher temperatures the composition of the solution with a maximum viscosity approaches to 33·3 mol. %, corresponding to the hydrate $C_2H_5OH + 2H_2O$.

Most dilute aqueous solutions are characterised by a positive $d \log \eta/dc$ decreasing with increasing temperature. The same behaviour may be expected for dilute solutions in alcohols.

For mixtures of non-associated liquids, for instance ethyl ether (viscosity 0·00230 at 25°) and carbon disulphide (viscosity 0·00358 at 25°), $\log \eta$ is, in general, a linear function of the molecular concentration, and therefore a constant value is found for $d \log \eta/dc$ in the whole range from $c = 0$ to $c = 100$. If c represents the concentration of the more viscous component θ is positive; but if c represents that of the less viscous component θ acquires a negative value. Normally the absolute value of θ for such solutions decreases with increasing temperature.

9. *Associating substances.* Very different and much more complicated results are obtained if we investigate substances, which become dissociated through dilution, which often present maxima of viscosity at certain concentrations. A typical substance of this kind is ethyl alcohol, which consists of strongly associated molecules in a concentrated state, but, as the freezing point determinations indicate, of simple molecules, probably associated to a certain quantity of water, in very dilute aqueous solutions. I therefore give in the following table some determinations of Horiba [1910, 1912] on the viscosity of aqueous solutions of ethyl alcohol at 25°.

c	c_1	η	$\log \eta$	$\log \eta/c_1$	$\log \eta_2/c_2$
0	0	1	0	(0·0440)	−0·000847
3·65	1·46	1·151	0·0611	0·0418	−0·000240
7·84	3·224	1·339	0·1268	0·0394	0·000435
14·88	6·403	1·708	0·2325	0·0363	0·00158
27·49	12·92	2·344	0·3696	0·0286	0·00327
40·35	20·93	2·617	0·4178	0·0200	0·00423
54·56	31·96	2·552	0·4068	0·01273	0·00473
63·95	40·92	2·375	0·3757	0·00918	0·00493
69·25	46·84	2·233	0·3489	0·00745	0·00497
79·50	60·24	1·924	0·2842	0·00472	0·00502
98·33	95·8	1·276	0·1058	0·001104	0·00502
100	100	1·2153	0·0847	0·000847	(0·00502)

c is the concentration in per cent. of weight, c_1 the molecular percentage

of alcohol, $c_2 = 100 - c_1$ that of water. For the calculation of c_1 the molecular weights 46 for alcohol and 18 for water have been used.

The viscosity decreases when the dissolved molecules form greater aggregates. Therefore the quotient $\log \eta/c_1$ also decreases with increasing *concentration* c_1. The binding of water to the alcohol molecules would have an opposite effect, *i.e.* increase the viscosity on *dilution*, but the influence of the disaggregation predominates. If we regard the series from the other side, the water behaves in the same manner. The water molecules associate with each other when the concentration of water, c_2, increases. For this case we have to choose the viscosity of pure alcohol as unit, *i.e.* to put $\eta_2 = \eta/1{\cdot}2153$. The figure 1·2153 for pure alcohol is not given by Horiba but is calculated from the data of Thorpe and Rodger [1894]. We also see that $\log \eta_2/c_2$ decreases with increasing c_2. But this decrease is very small till we reach concentrations $c_2 = 100 - c_1$ of about 60 % (mol.). The two named effects here nearly compensate each other.

In similar cases, when chemical changes occur on dilution we are not entitled to expect $\log \eta/c$ to remain constant, when the concentration changes. Only for solutions so dilute that further dilution does not change their composition may such constancy be expected. For this case we have then to look for the value,

$$(d \log \eta/dc)_0 = \theta \qquad (2\,a)$$

where the index 0 indicates infinite dilution or concentration zero, as the characteristic constant θ. For alcohol dissolved in water θ is about 0·044 and for water dissolved in alcohol θ is about equal to 0·00502 if we calculate with molecular concentrations.

10. *Solutions of nitrocellulose.* This is precisely the manner in which Baker [1913] treated his data regarding the viscosity of solutions of nitrocellulose in different solvents. He expresses the concentration (c) in grams per 100 cc. of solution. As instance I give below Baker's Table II of solutions in acetone at 25°. I have supposed that the molecular weight of nitrocellulose is so great, that the number of its molecules may be neglected in comparison with that of the acetone molecules, and by means of the specific gravity I have calculated the number of grams of nitrocellulose in 100 g. of acetone. This number is written under c_1 and is very nearly proportional to the molecular percentage of the nitrocellulose. The viscosity of acetone at 25° is given as $\eta = 3{\cdot}15 \,.\, 10^{-3}$ C.G.S. units. This number is taken as unit for η in the table.

c	c_1	η	$\log \eta$	$\log \eta/c_1$
0	0	1	0	(2·2)
0·2212	0·2809	3·88	0·589	2·096
0·5261	0·6778	18·7	1·273	1·878
0·7922	1·008	56	1·748	1·734
1·058	1·349	143	2·160	1·602
1·640	2·094	711	2·852	1·358
2·348	3·008	5050	3·703	1·230
3·865	4·782	53300	4·727	0·949

Just as for alcohol the quotient $\log \eta/c_1$ sinks with increasing concentration and viscosity. But the quotient diminishes only in the proportion 2·2 : 1, when η increases in the proportion 1 : 50,000, whereas for the alcohol solution in water the same decrease of the quotient is followed by an increase in η only in the proportion 1 : 2·6.

Just as with the alcohol, it is generally supposed that nitrocellulose in a concentrated state consists of molecular aggregates, which are in this case very large. On solution in acetone or some other solvent these aggregates are partially broken down and in higher degree the greater the dilution. This explains why $\log \eta/c_1$ decreases with increasing concentration. It is evidently not possible to explain this behaviour by supposing that the nitrocellulose binds a part of the solvent, for in this case, and if the dissolved molecules do not alter their composition with concentration, the quotient $\log \eta/c_1$ would diminish with increasing concentration. Now Hatschek has tried [1916, 1] to calculate the quantity n of the solvent bound to 1 g. of nitrocellulose and found a negative value of about – 30 cc. We quite agree with him that this result "is devoid of any physical meaning." But therefrom to conclude that formula (2) has no physical meaning, as Hatschek does, seems the less cautious as Baker himself in calculating his experiments has on purely empirical grounds been led to the formula (2 *a*), derived from formula (2), which Baker seems not to have known. Baker's investigation may therefore be regarded as giving a very strong confirmation of formula (2), in direct opposition to Hatschek's opinion. Baker has found that the constant θ in formula (2 *a*) decreases with increasing temperature, which is characteristic of solutions of substances which form molecular complexes with the solvent. It is therefore probable that this very general feature also prevails for solutions of nitrocellulose, a conclusion to which Baker was also led from other considerations. This is also indicated through the contraction on dissolving "blasting soluble nitrocellulose" in acetone, when the total volume diminishes by about one-third of the volume of the dissolved substance.

The specific gravity of nitrocellulose is 1·634 according to Escales. If we recalculate the figures given by Baker for $d \log \eta/dc$, where c is expressed in grams per cc. and natural logarithms are used, so that we obtain the corresponding values for volume per cent. and in common logarithms, we get the value of θ in formula (2). In the case tabulated above Baker has the figure 6·22, which reduced in the said manner gives $\theta = 4{\cdot}42$, *i.e.* 406 times as great as the value demanded by Einstein's theory. The values given by Baker for different solutions fall between 3·22 and 10·45 corresponding to values of θ between 2·29 and 7·43 which are 210 and 684 times as great as the theoretical value calculated by Einstein.

11. *Solutions of rubber.* Hatschek has also used some determinations by Schidrowitz and Goldsbrough [1909, 1, 2] on rubber solutions for calculations by means of which he tries to attack formula (2). Hatschek uses the following figures regarding hard Para rubber in benzene. η is the relative viscosity, that of the solvent being taken as unit. The concentration c is given in grams per 100 cc. and these cannot be recalculated to cc. in 100 cc. benzene, because "for the rubber solutions the densities are not available." The value of $\log \eta/c$ is therefore "approximate only but in view of the low concentrations the possible error is certainly slight."

c	η	$\log \eta$	$\log \eta/c$	$\log \eta_{calc.}$	$\eta_{calc.}$
0·25	2·5	0·40	1·60	0·39	2·5
0·50	6·8	0·83	1·66	0·78	6·0
1·00	32·3	1·51	1·51	1·56	36·3

As $\log \eta/c$ is greater in the second case than in the first one, a calculation of n, *i.e.* the quantity of benzene bound to 1 g. of rubber, gives a positive value $n = 14{\cdot}4$. The inverse is true if we compare the second and the third measurement, they give $n = -21{\cdot}5$, which is evidently physically impossible. Against this it must be said that the whole calculation is illusory, as the magnitude of the experimental errors is not considered. From the measurements of Kirchhof [1914] quoted below it is very probable that these errors may reach 10 or 12 % and, as the figures given above show, it is possible to reconcile these data of Schidrowitz and Goldsbrough with the demands of formula (2) within the said limit of errors. Hatschek in his calculations uses logarithms with five decimals, which correspond to an accuracy of 0·003 %. It is therefore not to be wondered at that his results become highly "erratic."

Kirchhof has given a great number of measurements on the viscosity at 20° of solutions of indiarubber in different solvents. The concentration ranges between 0·5 and 3 % (g. in 100 cc.) and the viscosities are expressed

in that of the corresponding solvent as unit. His results are given in the following table, corresponding to Kirchhof's Table IV. The data in the table are relative viscosities, observed and calculated by means of formula (2). Kirchhof also gives no specific gravities of his solutions, the calculation is therefore only approximate as in the preceding case.

Solvent	0·5 %	1 %	2 %	3 %
Gasolene, obs.	1·9	4·3	—	94·0
calc.	2·1	4·47	—	89·1
Benzene, obs.	2·1	4·7	23·5	97·3
calc.	2·16	4·7	21·9	102·2
Carbon tetrachloride, obs.	2·6	7·5	—	211·3
calc.	2·45	6·03	—	219
Tetrachloroethane, obs. ...	2·5	6·9	—	168
calc....	2·40	5·76	—	191
Pentachloroethane, obs. ...	3·0	8·7	46·0	284·5
calc....	2·6	6·76	45·7	309

The last figure for pentachloroethane is given by Kirchhof as 213·5 but a recalculation of the original observations in Table I and Table IV gives 284·5.

Kirchhof has made experiments with different pressures from 10 to 60 cm. of water. For distilled water he has found the times of flow to be 50·6 seconds if the pressure was 10 cm. and 10·0 seconds if it was 60 cm. As the times of flow are supposed to be strictly proportional to the viscosity they ought to be in inverse proportion to the pressures and the first figure should be 60 sec. if the second figure is right. Still worse is the agreement for gasolene where the time of flow is 32·2 sec., instead of 45·6 sec., and for benzene 28·6 instead of 40·8. The last figures, 45·6 and 40·8 respectively, are six times the time of flow at a pressure of 60 cm. water. This great disagreement, reaching in the three cases 18·6, 38·5 and 44·2 %, depends upon the very short time of flow at the higher pressure (10·0, 7·6 and 6·8 seconds). It is very much to be regretted that Kirchhof has preferred just these high pressures (60 cm. water) for the determinations given in his Table IV, reproduced above. It must therefore be said that the calculated figures agree with the observed ones within the errors of observation, although the difference in one case is nearly 25 % (this may of course be due to an accidental error). The figures of Schidrowitz and Goldsbrough do not contain any data from which the errors of observation might be estimated, but they very much resemble those of Kirchhof. Therefore I find it possible or even probable that, as said above, their figures agree satisfactorily with the demands of the logarithmic formula, especially as they characterise their experiments as "preliminary."

If we now calculate the values of θ in formula (2) for these substances we find:

	θ	A
Hard Para rubber in benzene ...	1·435	132
Indiarubber in gasolene	0·618	57
„ „ benzene	0·637	59
„ „ carbon tetrachloride	0·74	68
„ „ tetrachloroethane ...	0·722	67
„ „ pentachloroethane	0·788	73
„ „ trichloroethylene ...	0·584	54

The specific gravity of the rubbers is not given, it is supposed to have been 0·95. Below A is written how many times the θ values exceed the theoretical value deduced from formula (1). This ratio is not as great as for the solutions of nitrocellulose, but still they are amongst the largest observed.

12. *Hatschek's formula for solvatation* (*hydratation*). Hatschek [1911, 1912, 1, 2] has given a formula for calculating the quantity of solvent, which is bound to 1 g. of the dissolved substance. The unbound quantity of the solvent is supposed not to exceed 70 % of the total volume. The formula is very simple, it has the form:

$$\eta_0/\eta = 1 - \sqrt[3]{\phi} \quad \text{.....(3)}$$

where η, as usual, is the viscosity of the solution, η_0 that of the solvent and $100\,\phi$ is the volume per cent. of the dissolved substance with its associated solvent. Regarding this formula Smoluchowski says: "It may be an interpolation formula of a certain use in many cases, but it is probably of no general signification, as it is impossible to give it a rational foundation." Till now its practical application has been very scant. Hatschek gives himself [1916, 1] an example when he says: "If the association factor is calculated, say for the 0·5 % concentration of the first example (0·5 % Para rubber in benzene $\eta = 6{\cdot}8$; cf. § 11) we find that about 62 % of the total volume of solvent is already associated with the solute. A 1 % solution would therefore be impossible if the association factor were constant. Therefore the association factor must be lower for the 1 % solution. There is certainly nothing inherently improbable about decreasing association with increasing concentration." Now we must confess that the formula has a very reduced interest if it does not give a constant association factor. Hatschek has himself emphasised the high value of Miss Chick's calculations, because his formula gives nearly constant results for the association factor (cf. below).

Kirchhof has used formula (3) for calculating his figures and has not observed the difficulties which occur in nearly all the results. A glance at the figures in his Table V gives no good prognosis for the use of formula (3).

The results of Miss Chick, which are regarded by Hatschek as the best proof of his formula, are considered by herself to be very encouraging. I give below a typical example, the calculations regarding the viscosity of pseudo-globulin. c is the concentration in per cent. of weight, η the relative viscosity in regard to the solvent water, B is the volume occupied by 1 g. dissolved substance as hydrate calculated according to formula (3) and said to be nearly constant, when the volume, p, of the hydrated molecules exceeds 50 % of the total volume—p is given in per cent. of the total volume.

c	η	p	B	
3·61	1·40	2·3	0·64	
5·95	1·85	9·7	1·603	
8·32	2·59	23·2	2·716	
10·04	3·43	35·6	3·441	
11·82	4·70	48·8	3·990	
14·06	8·05	68·8	4·585	
16·43	13·74	79·7	4·627	Mean value 4·50
18·45	23·31	87·6	4·499	(4·496)
20·37	38·79	92·4	4·272	

The formula should be valid if p exceeded about 50 %. Instead of that we find that B, which ought to be constant, increases strongly until p reaches about 80 %. This increase is also very pronounced from $p = 50$ % on, which corresponds to the interpolated value $B = 4{\cdot}06$, although Miss Chick says that "the value v/c ($= B$) remained constant when the disperse phase occupied more than one-half the total volume." At $p = 74{\cdot}06$ is a peculiar point, for if the hydrated molecules are spherical, they are tightly packed there, so that the spheres touch each other on all sides. Above this point formula (3) cannot be valid. We find that at higher values of p this deficiency of the formula is manifested by a decrease of B with increasing concentration. At higher concentrations than $c = 20$ % B would decrease still more rapidly. Through this circumstance the function B passes through a maximum, in the neighbourhood of which its value is approximately constant, and this is then said to be a proof that the formula is correct. I think it would be more justifiable to say that the formula gives unreliable results in the neighbourhood of the said point. According to this it seems clear that Smoluchowski has overestimated the practical value of formula (3). The hyperbolical form of equation (3) makes it unfit to represent the regularly continuous course of η with concentration.

13. *Viscosity of rice-starch suspensions.* From Odén's investigations on colloidal sulphur it is evident that the viscosity of its solutions

increases more rapidly with concentration than according to a linear formula:

$$\eta = \eta_0 (1 + k\phi) \quad \text{.....(1 } a\text{)}$$

where k is a constant, which according to Einstein ought to be 2·5. Hatschek [1910, 1911, 1913, 1916, 2] has also deduced a similar formula, in which k has the value 4·5. Regarding this deduction, Smoluchowski makes the remark, that "it is erroneous (unrichtig) and consequently the formula, with $k = 4{\cdot}5$, lacks any general signification." Hatschek believed that the discrepancy between his formula and Odén's experiments depended upon disturbances due to the use of capillary viscosimeters in the determinations. He therefore made some experiments in collaboration with Miss Edith Humphrey [1916] with a modification of the viscosimeter of Couette. The substance examined was rice-starch in granules of 0·003 mm. diameter, suspended in a mixture of toluene and carbon tetrachloride. The calculated viscosities showed a very great dependence on the velocity of rotation of the instrument. The calculated viscosity of the solvent increased by about 10 %, when this velocity increased in the proportion 1 to 5. With the suspensions the inverse was true, a 6 % suspension diminished its apparent viscosity by about a third for the said increase of velocity. Further a kind of hysteresis took place, so that the viscosity of the solvent seemed to be about 5 % greater if the velocity decreased, than if it increased. Evidently this cannot be right for mixtures of such liquids as toluene and carbon tetrachloride—for coagulating solutions a kind of hysteresis in the opposite direction is often observed; cf. § 14. It must be said that the apparatus used was far inferior to the capillary viscosimeter. Hatschek is of the opinion that the measurements with the highest velocity used, 125° per second, are the most reliable. He has given the results in curves and I have taken the following figures from measurements on the curve signed 125 of his fig. 3.

Volume %		Relative viscosity			
given	corrected	H.	measured	calculated	Difference %
0	0	1	1	1	—
2	2·041	1·3	1·288	1·275	−1·0
4	4·167	1·6	1·647	1·642	−0·3
6	6·384	2·1	2·109	2·137	+1·3

The corrected value of the volume per cent. is expressed in the volume of the solvent taken as 100, in order that it should be proportional to the molecular concentration. Below H. are written the figures estimated by Hatschek. From these figures he concludes that the linear formula (1 a) is valid up to

4 % of volume, but not to 6. It is clear that, if more than one decimal had been used, this assertion would have proved to be false unless errors of observation as great as 3 % are admitted. But under such circumstances the accuracy is quite insufficient for examining the validity of formula (1 *a*). On the other hand we learn from the last two columns that the logarithmic formula (2) represents the observations with a much higher accuracy, and well within the limits of the errors of observation. It must therefore be said that these experiments confirm the observations of Odén, which also obey the logarithmic formula, instead of showing them to be incorrect. The same cannot of course be said regarding the linear formula (1 *a*).

θ in formula (2) is in this case 0·0517, *i.e.* 4·76 times greater than the value demanded by Einstein and 2·7 times greater than that expected by Hatschek.

14. *Irregularities.* In working with emulsions with a tendency to coagulate we meet with many difficulties. Such a case has been investigated by Freundlich and Ishizaka [1913]. They prepared a solution of $Al(OH)_3$ according to the method of Crum and coagulated it by means of $K_4C_6N_6Fe$. m designates the quantity of $Al(OH)_3$, expressed in grams of Al_2O_3 per litre, η the relative viscosity at 25°.

m	η	log η	log η/m	$\eta_{calc.}$	A
0·187	1·154	0·0622	0·333	1·135	543
0·28	1·231	0·0902	0·322	1·210	526
0·48	1·408	0·1485	0·310	1·386	507
1·12	2·069	0·3158	0·282	2·140	461

As seen from these figures log η is nearly proportional to the concentration m and the calculated values of η agree tolerably well with the observed values. But there is a pronounced tendency of log η/m to decrease with increasing concentration. The coagulating solution looks, to begin with, just like the uncoagulated, but its viscosity increases slowly, and at first after several hours, if the solution is not too concentrated, the flocculation becomes visible. It is supposed that the maximal viscosity is reached before this point, but that is not at all proved and in all cases it is probably rather difficult to determine this maximal viscosity, which is tabulated above under η. Further this viscosity is, for more concentrated solutions, dependent on the mechanical treatment. Thus the maximal viscosity 2·069 of the last solution changes to 1·62 after shaking. Under such circumstances it is clear that deviations between the observed and the calculated values may occur. The maximal viscosity depends also in a high degree on the coagulating power of the salt added as is seen from the following table for different salts of potassium or ammonium. The solution used was always the same with $m = 0{\cdot}88$.

Coagulating salt	η	Potassium salicylate	1·929
Potassium nitrate	1·423	,, sulphate	1·998
,, thiocyanate	1·423	,, ferrocyanide	2·000
Ammonium chloride	1·481		

From the specific gravity, 3·85, of Al_2O_3 I have calculated the specific gravity of $Al(OH)_3$ under the assumption that no change of volume takes place on hydration. With the figure 2·21, found in this way, I have calculated how many times, A, the θ-value for 1 vol. % $Al(OH)_3$, exceeds the theoretical figure 0·01086. As seen, A reaches the value of about 5·00. Probably the floccules correspond to a much higher degree of hydration than that expressed by the formula $Al(OH)_3$, so that A should be considerably diminished.

Similar remarks may be made regarding some determinations of Bingham and Durham [1911], in which small additions of potassium chloride increased the viscosity of suspensions of infusorial earth, in one case by 60 %, or diminished that of a suspension of clay by about 37 %, whereas one drop of acetic acid increased the viscosity of a graphite suspension by 81 %. The influence increases with the concentration of the suspension. Evidently we have here to do with a change in the state of flocculation. These suspensions do not at all follow the formula (2) for higher concentrations. Probably the flocculation makes the suspended particles cling to the walls of the viscosimeter tube, thereby narrowing it. We must therefore use formula (2 *a*). In this case it is sufficient to calculate A from the values of not too concentrated suspensions. For infusorial earth (3·2 vol. %) A increases with temperature, as is seen from the following table:

Aqueous suspensions		Alcoholic suspensions	
Temp. ° C.	A	Temp. ° C.	A
25	3·03	30	2·59
45	3·37	45	2·83
65	3·74	65	3·97
75	3·82		

For china clay (in 2·48 % suspension) the corresponding figures are:

Temp. ° C.	A
35	6·33
45	6·28
65	6·70

For graphite (Acheson's "aquadag," 0·4 vol. %) the figures are rather irregular and give a decrease of A, from $A = 7·12$ at 30° to $A = 6·55$ at 65°.

For solutions of indiarubber the irregularities are perhaps still greater than in the last named cases as Fol [1913] has shown. The viscosity of a solution diminishes, when it is allowed to flow repeatedly through the tube of the viscosimeter. This corresponds to the behaviour of the suspensions of $Al(OH)_3$ on shaking. But if we make a solution of a given quantity of rubber in a given quantity of benzene and shake this solution, its filtrate, taken after 24 hours, has a greater viscosity than if it had not been shaken. This depends upon a disintegration of small solid particles on shaking, so that they pass through the filter. But the increase of the viscosity is greater than corresponds to the increased concentration. This peculiarity is probably connected with the circumstance that the parts of the rubber which enter at first into solution give a lower viscosity than the parts dissolved later on. The viscosity of a rubber solution diminishes on standing.

From what has been said it seems evident that it is necessary to use the greatest precaution if we wish to draw theoretical conclusions from measurements on the viscosity of substances which exhibit such irregularities, specially when the measurements were made at a time when these irregularities were much more imperfectly known than they are to-day.

15. *The values of k in formula* (1 *a*). We have seen above that Bancelin's experiments gave a brilliant confirmation of Einstein's theory, demanding $k = 2{\cdot}5$. Further the experiments of Mifka are well in concordance with this theory. But all other experiments on colloidal solutions, quoted above, give too high values of k.

To begin with, the experiments of Odén for colloidal sulphur give the following values:

For submicroscopical solutions (diam. of granules $100\mu\mu$)	at	$5°$, $k = 3{\cdot}60$.
" " " " " "		$20°$, $3{\cdot}67$.
" " " " " "		$40°$, $3{\cdot}88$.
For amicroscopical solutions (diameter $10\mu\mu$)	at	$25°$, $4{\cdot}73$.

For all the other suspensions or emulsions examined (with exception of serum albumin, an exception perhaps due to errors of observation) the values of k have been found to exceed 2·5, in many cases a hundredfold and more. The question arises: what is the cause of this discrepancy? Smoluchowski has pointed to the probable cause. He says: "For the sphere this value (k) has evidently a minimum value, as it by virtue of its rolling movement has influence upon the smallest possible volume of the liquid in its state of shearing." Therefore the coefficient 2·5 in Einstein's formula applied to

colloidal solutions must be regarded as a minimum value. But there is evidently no sufficient ground to replace it by a higher value as Hatschek proposes.

There is another peculiarity, which Smoluchowski has not observed, namely that k in formula (1 a) decreases with increasing diameter of the spherical particles, as is seen from Odén's measurements. Now we know that the Brownian movement of the suspended particles increases, when their dimensions are diminished. Therefore the same quantity of smaller particles will come in contact with a great number of molecules of the solvent through their speedier movement, than the same quantity of bigger particles. This circumstance will also increase the value of A when the dimensions of the particles decrease.

On the same ground, as the Brownian movement increases with temperature, the value of A or of k will increase with temperature. This effect is very pronounced in Odén's figures. It is due not only to the movement of the suspended particles, but also to that of the molecules of the solvent. This must be true for particles of any form and it is seen from the experiments on suspensions of clay and ground infusoria that this statement is true. When molecular aggregates of the solvent are formed their disaggregation with increasing temperature has the inverse effect.

For substances which are hydratated θ decreases with rising temperature. This depends upon the partial dissociation of the hydratated molecules with increasing temperature, whereby the volume concentration of the dissolved or suspended "molecules" diminishes. This seems to be the case with most aqueous solutions and Miss Chick has observed it for the solutions of protein substances. The low value of $k = 2{\cdot}14$ for serum albumin is probably due to errors of observation, which have caused a too high value of the hydratation factor, n. The decrease of A with increasing temperature for graphite suspensions is perhaps due to a diminished agglutination of the particles, for it is not probable that graphite forms hydrates.

The hydratation has a great effect on the viscosity at higher concentrations and causes positive deviations from formula (2), if we calculate the values as if no hydratation took place. We have not found any instance of such positive deviations for solvents which are non-associated in contradistinction to the highly associated water. Therefore it is not probable that these non-associated solvents in the cases examined hitherto, especially with solutions of india-rubber or nitrocellulose, enter into molecular aggregates with the dissolved molecules, as is maintained by Hatschek on the ground of calculations by

means of his formula (3). But probably many alcoholic solutions behave as aqueous ones. On the other hand suspensions of infusorial earth in alcohol, as well as similar suspensions of infusorial earth, clay or graphite in water show a θ-value, that increases with temperature. In this case we have no hydratation nor alcoholatation of the suspended particles. The very high viscosity of these suspensions in greater concentrations, which is followed by a very pronounced deviation from formula (2) depends on a formation of large aggregates between the suspended particles themselves, which form a kind of felt which obstructs the capillary tube. Similar cases will probably be very common in agglutinating or coagulating suspensions in higher concentrations.

The very large values of θ or A found in most cases have a similar explanation. Every chemist knows fairly well the voluminous flocculi of hydrated alumina which on desiccation crumble to nearly insensible granules. It is quite natural to suppose that the high value of A, about 500, in this case depends on this voluminous nature. The hydrated particles may further, as Smoluchowski says, possess a spongy structure or be formed as spherolites consisting of very fine needles radiating from a centre. The high values of A found for solutions of indiarubber or nitrocellulose suggest a similar form for the corresponding aggregates, which may also cling to the walls of the viscosimeter tube and cause similar deviations from formula (2) as for suspensions of clay or infusorial earth or graphite. Many examples of such a behaviour in more concentrated solutions are found in the memoirs of Schidrowitz and Goldsbrough. This behaviour seems very probable for indiarubber in the dissolved state, the solutions being extremely sticky. Regarding the similar property of moist clay, on which its high plasticity depends, Le Chatelier [1914, p. 481] has drawn attention to the circumstance that it is a consequence of the fine laminated structure of kaolin. The same may be true for graphite which crystallises in thin hexagonal scales. Probably its high lubricating power is connected with this property. Evidently we have here to do with capillary forces which attain a predominant influence for laminated substances in the form of very fine dust.

Hatschek has also made the objection against formula (2) that it has no theoretical foundation. Lees [1900, 1901] has given such a one (cf. also § 5). But it is also to be remembered that the most "rational" laws have originally been built up from purely empirical foundations.

16. *Summary.* The formula of Einstein, as applied to colloidal solutions, represents a limiting law. It has been confirmed by the experiments of

Bancelin. But in most cases the viscosity is much higher than this law demands. The anomalies depend on the Brownian movement, on the non-spherical form of the suspended particles, on formation of aggregates with the solvent especially on hydratation or on sticking together of suspended particles, circumstances which all increase the viscosity. The logarithmic formula (2) is found to be valid for true solutions as well as for pseudosolutions (emulsions and suspensions). Deviations from this formula are caused through chemical changes occurring on dilution.

The chemical changes occurring on mixing solvent and solute are the following:

(1) The solvent may be associated and its complex molecules may be dissociated by the addition of the solute. This process is followed by a diminution of the viscosity. A rise of temperature exerts also such a dissociating influence, whereby a diminution of θ in formula (2) results.

(2) A dissociation of the molecules of the solute has an opposite influence, so that the viscosity increases thereby. This corresponds to a higher viscosity of suspensions with small spherical granules, compared with that of a similar suspension with larger granules. If the suspended particles possess a laminated structure, they form felty aggregates, which in higher concentrations raise the viscosity.

(3) Association between molecules of the solute and of the solvent increases the viscosity. In this case the dissociating influence of rising temperature diminishes θ in formula (2).

Objections made against the validity of the logarithmic formula, raised in this journal by Hatschek, do not bear a close analysis. Experimental attempts by Hatschek and Miss Humphrey to disprove this formula have wholly failed. Hatschek's formula for calculating the degree of binding between solvent and solute not only lacks theoretical foundation but is also incompatible with experimental facts.

REFERENCES

Arrhenius (1887). *Zeitsch. physikal. Chem.* **1**, 287.
—— (1916). *Medd. Vetensk. Nobelinstitut*, **3**, No. 13.
Baker (1913). *J Chem. Soc.* **108**, 1653.
Bancelin (1911, 1). *Compt. rend.* **152**, 1382.
—— (1911, 2). *Kolloid Zeitsch.* **9**, 154.
Bingham and Durham (1911). *Amer. Chem. J.* **46**, 278.
Chick (1914). *Biochem. J.* **8**, 261.
—— and Lubrzynska (1914). *Biochem. J.* **8**, 59.
Einstein (1906). *Ann. Phys.* (4), **19**, 289.
—— (1911). *Ann. Phys.* (4), **34**, 591.
Equér (1917). *Medd. Vetensk. Nobelinstitut*, **3**, No. 22.
Fol (1913). *Kolloid Zeitsch.* **12**, 131.
Freundlich and Ishizaka (1913). *Kolloid Zeitsch.* **12**, 230.
Hatschek (1910). *Kolloid Zeitsch.* **7**, 301.
—— (1911). *Kolloid Zeitsch.* **8**, 37.
—— (1912, 1). *Kolloid Zeitsch.* **11**, 280.
—— (1912, 2). *Kolloid Zeitsch.* **11**, 284.
—— (1913). *Kolloid Zeitsch.* **12**, 238.
—— (1916, 1). *Biochem. J.* **10**, 325.
—— (1916, 2). *Proc. Phys. Soc.* **28**, 250.
Horiba (1910). *J. Tokio Chem. Soc.* **31**, 922.
—— (1912). *Tables annuelles de constantes*, **1**, 33.
Humphrey and Hatschek (1916). *Proc. Phys. Soc.* **28**, 274.
Kendall (1913). *Medd. Vetensk. Nobelinstitut*, **2**, No. 25.
Kirchhof (1914). *Kolloid Zeitsch.* **15**, 30.
Le Chatelier (1914). *La silice et les silicates* (Paris).
Lees (1900). *Phil. Mag.* (5), **49**, 225.
—— (1901). *Phil. Mag.* (6), **1**, 130.
Mifka (1911). *Sitzungsber. Akad. Wien, Math.-Nat. Kl.* Abt. II a, **120**, 1173.
Noack (1886). *Ann. Phys. Chem.* **27**, 289; **28**, 666.
Odén (1913). *N. Acta R. Soc. Upsala* (7), **3**, No. 4, 103.
Schidrowitz and Goldsbrough (1909, 1). *J. Soc. Chem. Ind.* **28**, 3.
—— (1909, 2). *Kolloid Zeitsch.* **4**, 226.
Smoluchowski (1916). *Kolloid Zeitsch.* **18**, 190.
Thorpe and Rodger (1894). *Phil. Trans.* A. **185**, 397.
Woudstra (1908). *Zeitsch. physikal. Chem.* **63**, 619.

XIV. THE ESTIMATION OF SUCCINIC ACID.

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(*Received May 17th, 1917.*)

The original method employed by Pasteur for the separation of succinic acid from other acids produced during fermentation consisted in extracting the calcium salts with a mixture of alcohol and ether. The calcium succinate is insoluble and the salts of certain other acids dissolve. The method as now usually employed consists in adding to a concentrated solution of the calcium salts in water, sufficient alcohol to bring the final concentration to 85 %. The amount of succinate is estimated from the determination of the calcium in the precipitate.

The ordinary method is liable to serious errors, and probably the experience of inconstant results has led certain authors to discard it in favour of a method based on the use of some other salts such as the salts of barium. As a matter of fact however the errors to which the Pasteur method, as generally used, is liable, do not arise from any unsuitability of the calcium salt, but from other causes which are as follows:

(1) Loss of succinic acid during concentration. Succinic acid volatilises even from a solution which is alkaline to phenolphthalein. The loss may easily be 7 %.

(2) The solvent action of 85 % alcohol containing traces of peptone on calcium succinate. 0·15 g. of peptone in 100 cc. of the solution may introduce an error of 10 to 15 %.

(3) The presence of any substance which prevents the crystallisation of the calcium succinate.

(4) Acidity of the alcohol employed. The alcohol must be neutral.

(5) Loss through washing the precipitate.

The freshly formed precipitate of calcium salts is flocculent and in the presence of certain impurities such as peptone this condition persists. In the absence of impurities the precipitate gradually becomes crystalline. This change may be taken as an indication that the separation is quantitative. The errors referred to above may be avoided by attending to the following details.

In the case of the fermentation products studied by the writer, lactic acid is the only acid which remains to be separated from succinic acid after the volatile acids have been removed, but the method described here applies equally well to cases where other acids are present, provided the calcium salts of such acids are soluble in 85 % alcohol.

The mixture of succinic and lactic acids obtained by extracting the acidified solution of the non-volatile products of fermentation by ether, in a suitable continuous extraction apparatus, is dissolved in about 100 cc. of hot water and chalk added in excess. The solution should not be boiled as loss of succinic acid will occur by volatilisation. The neutralisation takes place slowly owing to the presence of the anhydride of lactic acid.

The final neutralisation is effected by adding a solution of calcium hydroxide after the addition of phenolphthalein. The solution is not neutralised from the start by lime water to avoid excessive dilution. The solution is kept hot during at least an hour to ensure the thorough conversion of the anhydride into calcium lactate, and the solution, distinctly pink, is filtered into a graduated flask of 200 cc. capacity. Of the filtrate 50 cc. is used to estimate the calcium corresponding to the total acids, *i.e.* succinic plus lactic and possibly other acids, the calcium being precipitated as oxalate and the oxalic acid equivalent of the calcium determined by titration with a standard solution of potassium permanganate. A suitable portion of the remaining solution is concentrated slowly to a volume of 13 cc.

This is done in a 110 cc. graduated flask in an oven kept at a little over 100°. Five or six hours are required for the concentration of 50 cc. of the solution. The author employs a series of tared 110 cc. graduated flasks and continues the evaporation until the flasks contain the required 13 g. of water. Alcohol is then added to make up the volume to 110 cc. which in the case of 96 % alcohol brings the final concentration to 85 %. The flask is well shaken and allowed to stand until the flocculent precipitate has become crystalline. This may require till next day but takes place in a few hours if the solution is free from impurity. The solution is filtered into a 100 cc. graduated flask, and after removal of the alcohol, the calcium is estimated in

this 100 cc. This calcium figure multiplied by 11/10 gives the amount of calcium corresponding to the acids of the soluble calcium salts, and the amount of succinic acid is obtained by subtracting the figure thus obtained from that previously found for the total acidity. The error of the experiment need not exceed 1 % of the weight of succinic acid present.

Should the amount of acids present be small, the estimation of the total calcium need not be made on a separate sample but after filtering the 100 cc. of the alcoholic solution the remaining 10 cc. of alcohol together with the precipitate of calcium succinate may be dissolved in water and the calcium determined. From this figure the amount of calcium corresponding to 10 cc. of the alcoholic solution is deducted, the difference representing the calcium in the precipitate[1].

The following example of the separation of a mixture of lactic and succinic acids may be given.

A mixture was made of 40 cc. of a solution of lactic acid (corresponding to 59·4 cc. N/10 lactic acid) and 30 cc. of a solution of succinic acid (corresponding to 26·25 cc. N/10 succinic acid) and the 70 cc. was extracted in a continuous apparatus by ether during nine hours. The extracted acids were treated as described above and the volume of the solution of the calcium salts made up to 200 cc. 50 cc. of this solution was brought to the boil and precipitated by oxalic acid. The calcium oxalate precipitate required for oxidation 21·1 cc. N/10 permanganate solution. The total acidity found therefore corresponds to 84·4 cc. instead of 85·6 cc. There is here an error of 1·5 %.

Another 50 cc. was evaporated to 15 cc. in a tared graduated flask and the volume made up to 110 cc. with 98 % alcohol. 100 cc. of the filtrate after removal of alcohol was precipitated by oxalic acid and yielded calcium corresponding to 13·15 cc. N/10 permanganate solution. This corresponds to 14·45 cc. for 110 cc. of the alcoholic solution. The total lactic acid is therefore 57·8 cc. N/10, which figure deducted from 84·4 gives 26·6. The amount of succinic employed was 26·25.

[1] *Note on the estimation of calcium by the permanganate method.* It is necessary for me to point out that the procedure which certain authors (Cahen and Hurtley, *Biochem. Journ.* 1916, **10**, 308) have adopted in connection with the well-known method of estimating calcium oxalate by permanganate is to be strictly avoided. The correct method of procedure is to filter the calcium oxalate upon asbestos in a suitable vessel, such as the ordinary Gooch crucible, and, after washing it, to decompose it by sulphuric acid in the ordinary way. The washing takes a few seconds and there is certainly no need for the seven washings of which these authors speak. But whatever procedure is adopted for collecting and washing precipitates, there can be no justification for boiling paper with sulphuric acid solution and titrating this with permanganate. Nothing must be oxidised except the oxalic acid.

This shows that all the manipulations can be carried out with an error less than 2 %.

The actual separation of the two acids need not involve an error of more than 1 %, as will be seen from Appendix, Ex. 2 (4, 5, 6).

Appendix.

Experiments demonstrating the magnitude of the errors referred to in the communication.

Ex. 1. Loss of lactic and succinic acids during the evaporation of the slightly alkaline solutions of the calcium salts.

Pasteur in describing the original method indicates the necessity of slow evaporation to avoid loss of succinic acid. It seemed to the writer of interest to know whether this loss occurred even when the solution was kept alkaline throughout the evaporation.

200 cc. of a solution of calcium lactate corresponding to 55·2 cc. N/10 acid, was evaporated on the water bath in an open vessel, the solution being kept alkaline throughout by the addition from time to time of a solution of calcium hydroxide; the dried residue was dissolved in water and the solution filtered from calcium carbonate. The filtrate contained calcium corresponding to 52·75 cc. of lactic acid. This represents a loss of 2·45 cc. N/10 lactic acid, or approximately 5 %.

Under the same conditions 36·6 cc. N/10 succinic acid lost 2·85 cc. or 8 % of the succinic acid employed.

To avoid such errors the evaporation should be made as directed in this communication in a long necked graduated flask in an oven regulated so that the temperature of the air is about 110°; under these circumstances there is no loss.

Ex. 2. The effect of peptone.

Peptone may introduce a grave error. It prevents the precipitation of calcium succinate by 85 % alcohol. Unneutralised peptone will dissolve calcium succinate readily, but the experiments here described refer to peptone which has been neutralised by boiling with chalk.

Into each of six tared graduated 110 cc. flasks were introduced 10 cc. of a solution of calcium lactate corresponding to 12·95 cc. N/10 lactic acid, and 10 cc. of a solution of calcium succinate corresponding to 6·85 cc. of N/10 succinic acid.

In addition

to No.	1	was	added	peptone	0·170 g.
,,	2	,,	,,	,,	0·023
,,	3	,,	,,	glycerol	0·70
,,	4	,,	,,	glucose	0·42

Absolute alcohol was added to each to make the volume up to 110 cc.

The calcium in solution in 100 cc. of the filtrate, was determined in each case.

No.	Substance added	$KMnO_4$ N/10 for 100 cc. of filtrate	Total calcium in solution	Calcium in precipitate corresponds to
1	Peptone, 0·17	13·8 cc.	15·1 cc.	4·7 cc.
2	,, 0·023	12·8	14·1	5·7
3	Glycerol, 0·70	12·2	13·4	6·4
4	Glucose, 0·42	11·75	12·9	6·9
5	Control	11·75	12·9	6·9
6	,,	11·90	13·1	6·7
		Succinic acid employed		6·85

This experiment shows that glucose does not affect the precipitation. There is a small effect due to glycerol, but the amount here added is about ten times that which would be present after a fermentation even if the whole of the glycerol were allowed to remain as an impurity. The error due to this cause is therefore negligible. On the other hand even 0·023 g. of peptone in 100 cc. of the fluid, may introduce an error of 15 % upon the amount of succinic acid estimated.

Summary.

By avoiding the errors described in this communication and following accurately certain details, succinic acid may be estimated in the presence of lactic acid, by the Pasteur method, with an error not greater than that of ordinary volumetric analysis.

In conclusion I beg to express my very best thanks to Professor Fernbach in whose laboratory this work was done.

XV. THE DISTRIBUTION OF THE FATTY ACIDS IN THE MILK FAT OF THE COW AND SHEEP.

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(Received June 1st, 1917.)

The variation in the character of the fat of cow's milk with advance of lactation, change of diet and other factors has received probably more attention at the hands of the investigator than any other aspect of the composition of milk. A careful examination of the formidable mass of literature in which this work is recorded reveals, however, that very little guidance has yet been obtained as to the fundamental causes underlying the changes observed. The prime reason for this comparative sterility of the work has undoubtedly been the difficulty of carrying out more than a superficial examination of the milk fat. No problem presents greater difficulties to the analyst than the quantitative separation and determination of a mixture of fatty acids, and the acme of difficulty is reached in the case of the most complicated mixture of fatty acids which is found in butter fat. For this reason investigators have mostly been content to limit their attention to the determination of various more or less well-defined groups of acids, such as steam-volatile, or water-soluble or unsaturated acids, etc., whereby naturally only a very imperfect record of the variations in composition has been obtained.

Efforts have not been lacking, however, to obtain a complete expression of the composition of the fat by the determination of each individual acid present, but in most cases the methods employed have been admittedly very imperfect, and in some cases assumptions have been made which subsequent research has proved to be incorrect. There is even a doubt whether the qualitative composition of butter fat has yet been fully explored, but there is clear evidence that the only acids present in more than minimal proportions are the unsaturated acid, oleic acid, and the eight saturated acids of the acetic series, representing the members with an even number of carbon atoms from C_4 to C_{18}, viz. butyric, caproic, caprylic, capric, lauric, myristic, palmitic, and stearic acids.

In the work reported in the present communication an attempt has been made to devise a method more precise than any previously used for the analysis of this complex mixture of acids.

Up to the present, most of the methods advanced for the quantitative analysis of this mixture have been based upon the difference in solubility of the several salts in certain solvents at given temperatures. Since these methods have proved more or less unreliable and have given discordant results in the hands of different workers we decided to examine the possibilities of a separation of the mixed methyl esters obtainable from butter fat. A detailed account of the method eventually devised will be found in the Experimental Part of this communication, and may be briefly summarised here.

A weighed amount of dry butter fat is converted to methyl esters by heating in ethereal solution with excess of methyl alcohol containing a small percentage of hydrogen chloride. The mixed esters are carefully separated from the reaction mixture and then fractionally distilled three or four times, in each case the distillation being made under atmospheric pressure until a temperature of 150°–160° is reached, and the fractionation subsequently completed under a pressure of about 15 mm.

The mixture of esters is thereby resolved into a series of fractions each assumed to contain only two saturated esters and one unsaturated ester, the latter being further assumed to be methyl oleate. Evidence in support of these assumptions will be found on a later page. From the iodine absorption value and the saponification value of each fraction it is then possible to calculate the weight of each ester contained in it (see later). Finally in summing up these results for the individual fractions the weight of each acid in the original weight of butter fat is arrived at.

In carrying out the fractional distillation under the conditions described in the Experimental Part it will be found that distinct fractions tend to collect round about the following temperatures:

Fraction	Boiling point	Fraction composed of
(*a*)	120°/760 mm.	Butyrate + Caproate
(*b*)	150°/760 mm.	,, ,, + a small proportion of oleate
(*c*)	70°/15 mm.	,, ,, + a small proportion of oleate
(*d*)	105°/15 mm.	Caproate + Caprylate + a small proportion of oleate
(*e*)	135°/15 mm.	Caprylate + Caprate + a small proportion of oleate
(*f*)	150°/15 mm.	Caprate + Laurate + a small proportion of oleate
(*g*)	175°/15 mm.	Laurate + Myristate + considerable oleate
(*h*)	202°/15 mm.	Myristate + Palmitate + a large proportion of oleate
(*i*)	210°/15 mm.	Palmitate + Stearate + a large proportion of oleate
(*j*)	over 212°/15 mm.	Stearate + Dihydroxystearate + a large proportion of oleate

The temperature registered by the thermometer during the distillation of the final fraction (*j*) varies considerably in different experiments, owing to the fact that this fraction is usually a small one, and that slight decomposition occurs during its distillation. If the esterification has been carried out successfully, however, no difficulty will be experienced in the distillation, and the decomposition involved will be so slight that no appreciable loss of ester will result. On an average, the *total* loss of esters incurred during an experiment amounts to less than 1 % of the weight of fat taken for analysis.

Haller [1906, 1907, 1908] states that the fractional separation by this method proceeds satisfactorily up to methyl laurate, but it was found impossible to obtain even the earlier fractions free from unsaturated material. Similar results have been recorded by Miss Smedley [1912] who concluded from a study of the iodine values of the various fractions, that unsaturated acids other than oleic acid exist in butter fat. In several instances she found that the iodine value of the fraction containing methyl caprate was appreciably greater than those of the fractions immediately preceding or following it, and accordingly the presence of a decylenic acid seemed probable, though she failed to isolate the compound. As we also repeatedly observed a local maximum iodine value for the fraction boiling at 120°/15 mm. (cf. p. 142) experiments were carried out with the object of identifying the unsaturated body present in this fraction. These, unfortunately, were inconclusive, the only positive result obtained being the isolation, from the product of an oxidation experiment with the fraction, of a small quantity of material, which from its crystalline appearance and melting point (130°) was regarded as dihydroxystearic acid.

Some indirect evidence of the possibility of methyl oleate being present in the lower boiling fractions, however, was obtained from the following experiment. Pure specimens of butyric, caproic, caprylic, capric, lauric, myristic, palmitic and stearic acids were obtained, and, before use, proved to be free from unsaturated material by the fact that no iodine absorption resulted when they were treated with Wijs' reagent. Weighed amounts of the above acids were then mixed with a known weight of pure oleic acid, so as to give a mixture of acids approximately the same as that occurring in butter fat. The acid mixture was then esterified in the usual way, the esters fractionated in the same manner as those prepared from butter fat, and after three fractionations the iodine value of each fraction was determined. In the following table are given details of

the final fractionation and the weights and iodine values of the individual fractions:

Fraction	Temperature °C.	Pressure mm.	Weight g.	Iodine value g.
1	65–130	760	0·29	1·867
2	–150	760	0·33	2·523
3	75– ?	15	1·27	1·730
4	–120	15	0·90	4·324
5	–140	15	1·18	5·823
6	–147	15	1·70	2·320
7	–155	15	2·90	2·840
8	165–170	15	8·61	3·630
9	–185	15	14·12	8·250
10	–200	12	14·77	39·12
11	–202	12	16·72	57·51
12	197–199	9	18·14	68·00
13	–205	9	6·80	72·61
14	Residue	—	1·80	71·50

As methyl oleate is the only unsaturated compound which can possibly be present, it is evident from the above results that it distils over with the lower esters. Further, a distinct local maximum iodine value is indicated corresponding to the fraction boiling between 120°–140° at 15 mm., so that the results, though at a lower level, are perfectly analogous to those obtained from an examination of butter fat. Accordingly it is reasonable to conclude that oleic acid is the only unsaturated acid present in the butter fat, and support is given to this view by the fact that so far all attempts to isolate or identify any other unsaturated body have proved unsuccessful.

If this be granted, it is possible, as indicated later (p. 151), to determine the mean molecular weight of the saturated esters present in each fraction: and these results, taken in conjunction with the observed boiling points for the respective fractions, point to each fraction containing, in addition to the proportion of methyl oleate, two saturated esters, one of these being present in large excess. Thus, for example, the saturated portion of the first fraction is largely methyl butyrate, a little methyl caproate being also present: the second fraction is largely caproate with a little butyrate or caprylate: and so on. Working on this principle, that there are present in each fraction, besides a proportion of methyl oleate, two saturated esters, one of which largely predominates, the amount of each ester present can be estimated from the mean molecular weight of the saturated esters by use of the following formulae:

$$x_1 = \frac{m_1}{m} \cdot \frac{m_2 - m}{m_2 - m_1} \cdot w \text{ and } x_2 = w - x_1,$$

where m is the mean molecular weight of the saturated esters in the fraction,

„ w is the weight of the saturated esters in the fraction,

„ x_1 „ „ „ ester, whose mol. wt. is m_1,

„ x_2 „ „ „ „ „ „ m_2.

By summing up the results obtained in this way for all the fractions, the total amount of each ester present is arrived at, and hence the percentage can be calculated.

Although the above method for arriving at the composition of the fat is liable to many sources of error, it has been found to yield consistent results when duplicate analyses of the same sample were carried out (see p. 145). This, of course, is no guarantee that the percentage of any particular acid found in a fat by this method is the correct one, but such was found to be the case when a more rigorous test was applied. When the artificial mixture of acids mentioned in the preceding pages was subjected to this method of analysis, the results obtained showed a remarkably close agreement with the theoretical values.

These are tabulated below for comparison:

Acid	Wt. of acid taken g.	Wt. of esters cal. from wt. of acid taken g.	Wt. of esters found by analysis g.
Butyric	4·34	5·03	5·026
Caproic	1·69	1·89	1·854
Caprylic	1·10	1·21	1·240
Capric	1·30	1·41	1·467
Lauric	3·80	4·06	3·994
Myristic	20·50	21·76	22·108
Palmitic	14·50	15·30	14·950
Stearic	1·26	1·32	1·416
Dihydroxystearic	—	—	0·210*
Oleic	40·00	41·98	41·675
	Total weight	93·96 g.	93·940 g.

* 0·21 g. dihydroxystearic ester would be produced from 0·188 g. oleic ester, therefore total weight of methyl oleate found = 41·863 g.

It would appear, from the above results, that the method is sufficiently reliable for all practical purposes for the detection of significant differences in composition between different fats. We have indeed already applied it for the comparison of the fat obtained from "first runnings" of milk with that from "last runnings" or "strippings" from the same cows. Reference to the summary on p. 145 will show that these two fats showed differences that were much larger than those obtained with duplicates of the same fat. The greatest differences noted are in the amounts of myristic, palmitic and

stearic esters, there being nearly 9 % less myristate in fat of "first runnings," but 6 % more palmitate, and nearly 3 % more stearate. The "first runnings" fat also yielded a larger proportion of methyl oleate, as was indicated by the iodine values.

Duplicate complete analyses of the fat obtained from ewe's milk gave quite a good agreement throughout (cf. p. 145), but the mean composition of ewe's milk fat thus arrived at showed marked differences from the average analysis of cow's milk fat, especially with regard to the percentages of the lower fatty acids. The amount of methyl butyrate was considerably higher than in cow's milk fat, the caproate somewhat lower, while the caprylate and caprate were between four and four and a half times as great. Without further evidence it is impossible to say whether these differences are characteristic or purely accidental.

With regard to the actual percentages of the several acids present in cow's milk fat, the results obtained by different observers vary within wide limits. Although this is only to be expected with a mixture such as butter fat, where so many factors contribute to the variation, still the great differences in the amounts, for example, of lauric acid, or stearic acid, reported as being present in butter fat, must be due mainly to the method of analysis employed. The following table (p. 145) gives a summary of results obtained by several workers, and there is added for comparison the results of the analyses, which are detailed in the present communication (pp. 147–162).

As far as the lower fatty acids are concerned the percentages found by us are about a mean of those hitherto published. The values for the butyrate tend to be a little high, and this is probably accounted for by the special precautions taken to secure efficient condensation when working with the lowest boiling fractions. The amounts of lauric acid found are considerably higher than was found by Browne, whose results are commonly quoted as authoritative. That butter fat does contain a considerable proportion of lauric acid was demonstrated by the following experiment. A fraction of methyl esters, obtained from butter fat, was found by the above method to consist of methyl caprate, methyl laurate, and methyl oleate approximately in the ratio 13 : 100 : 8. Three grams of this were hydrolysed, and the resulting acids isolated. The semi-solid mass obtained on cooling was spread on a porous plate, which soon absorbed the liquid oleic and capric acids, leaving behind fully two grams of a pure white solid acid. This weight agrees closely with the estimated amount of lauric acid present, and the solid after re-crystallisation from spirit was identified by its melting point (43°) as lauric

Composition of Butter Fat.

Observer	Butyric acid	Caproic acid	Caprylic acid	Capric acid	Lauric acid	Myristic acid	Palmitic acid	Stearic acid	Dihydroxy-stearic acid	Oleic acid
	%	%	%	%	%	%	%	%	%	%
Molt [1907, p. 281]			7·40		—	—	20·00	40·93	—	26·53
Koefoed [1891]	1·37	1·83	0·46	1·83	7·32	20·13	25·62	1·83	—	31·11
Bell [1890, p. 48]	6·13		2·09		—		49·46		—	36·10
Duclaux [1886]	3·38– 3·65	2·0– 2·26	—	—	—	—	—	—	—	—
Blyth [1903, p. 340]	3·49	2·40	0·80		—	—	47·50		—	40·40
Browne [1899]	5·45	2·09	0·49	0·32	2·57	9·89	38·61	1·83	1·00	32·50
Fleischmann and Warmbold [1907] — Sample 1 ...	5·00	2·00	0·15	—	—	—	52·12	—	—	35·36
Fleischmann and Warmbold [1907] — Sample 2 ...	5·00	2·00	0·15	—	—	—	51·10	3·35	—	33·30
Fleischmann and Warmbold [1907] — Sample 3 ...	4·32	2·16	0·67	—	—	4·46	48·94	5·54	—	28·81
Fleischmann and Warmbold [1907] — Sample 4 ...	4·32	2·16	0·67	—	—	10·00	42·75	2·00	—	33·00
Siegfeld [1907] — Sample 1 ...	3·53	2·50	1·03	—	—	25·35	17·18	—	—	42·75
Siegfeld [1907] — Sample 2 ...	3·58	1·26	2·87	—	—	22·94	20·84	—	—	40·53
Siegfeld [1907] — Sample 3 ...	3·27	1·73	1·89	—	—	30·70	16·89	—	—	37·98
Siegfeld [1907] — Sample 4 ...	3·35	1·68	2·21	—	—	26·00	20·96	—	—	38·09
Jensen [1905, I, 2]	3·92	1·88	—	—	—	—	—	—	—	—
Richmond [1914, p. 42]	3·43	3·25	0·51	1·77	6·94	19·14	24·48	1·72	—	33·60
Crowther and Hynd — Cow's milk fat — Dairy butter	4·45	1·45	0·99	1·10	3·55	20·13	15·24	1·08	0·68	45·47
Crowther and Hynd — Cow's milk fat — First runnings	4·30	1·98	1·11	1·51	5·08	10·38	17·47	5·93	0·30	46·49
Crowther and Hynd — Cow's milk fat — Last runnings	4·06	1·48	1·37	0·96	6·40	18·78	11·78	3·19	0·16	41·31
Crowther and Hynd — Cow's milk fat — Average	4·27	1·64	1·16	1·19	5·01	16·43	14·83	3·40	0·38	44·42
Crowther and Hynd — Sheep's milk fat — 1st analysis	6·60	1·06	4·18	4·72	4·73	13·20	13·87	4·56	0·36	40·61
Crowther and Hynd — Sheep's milk fat — 2nd analysis	6·48	1·29	4·27	4·86	4·67	14·28	12·81	4·22	0·18	40·72
Crowther and Hynd — Sheep's milk fat — Mean	6·54	1·18	4·23	4·79	4·70	13·74	13·34	4·39	0·27	40·67

acid. It seems from the above result that the usually accepted idea that only traces of lauric acid are present in butter fat must be modified. At any rate one cannot accept Caldwell and Hurtley's statement [1909], that the amount of lauric acid in butter fat is so small that none can be isolated from a pound. At the same time their observation, that palmitic acid is present in much smaller proportions than usually assumed, while myristic acid is more abundant, is borne out by our results, which in this particular also agree well with those of Siegfeld [1907]. It is with regard to stearic acid, however, that the greatest discrepancies have been reported. Many observers state that this acid either does not occur in butter fat, or is present only in very small amount. Thus Lewkowitsch [1913, p. 558] found only 0·49 % stearic acid in the insoluble fatty acids of butter fat, and Hehner and Mitchell [1896] also found very small proportions of stearic acid, varying from 1·3 to 3·6 %, and in some cases none at all. Koefoed [1891] and Browne [1899] both quote 1·83 % stearic acid, and the latter author writes:

"A noteworthy fact is the low percentage of stearic acid in butter fat.

This is somewhat contradictory to the statements which we find given by many authorities on the subject, who regard stearic acid as one of the principal acids in butter fat, it being placed by some writers as high even as 40 %. We believe that these statements should be modified somewhat; when we consider that oleic acid of molecular weight 282 constitutes over one-third of the insoluble acids from butter fat, it is self-evident that the percentage of stearic acid (mol. wt. 284) must be small in comparison with that of its lower homologues to give a mean molecular weight of 261 to the whole mixture....The low percentage of stearic acid should rank with the high percentage of soluble fatty acids, as one of the characteristic differences between butter and other animal fats."

In view of the above, it is surprising to find later evidence, which shows that the percentage of stearic acid in butter fat is considerable. Thus, Miss Smedley [1912] concluded that, of the total fatty acids from 10 to 15 % was stearic, and quite recently Holland, Reed and Buckley [1916] estimated that the proportion of stearic acid in the insoluble fatty acids from butter varied from 6·93 to 22·33 %, according to the type of fodder, and probably also to the individuality and period of lactation. Though the factors mentioned would, no doubt, influence the amount of stearic acid present, the variation seems extraordinarily well marked, and one feels inclined to doubt the applicability of Hehner and Mitchell's method to this particular analysis, especially as Lewkowitsch has pointed out that this method yields entirely unreliable or at best capricious results in a large number of cases where mixtures of stearic acid with acids other than palmitic and oleic are involved. Even if only palmitic and oleic acids be present, the results are still very uncertain; for example in the case of the fatty acids of cotton seed oil it was impossible to detect stearic acid by this method, even if 5 or 8 % of pure stearic acid had been added to the mixed fatty acids.

The average proportion of stearic acid obtained by us is 3·4 % for cow's butter fat, and 4·39 % for sheep's butter fat. The small amounts of dihydroxystearic acid found are regarded as oxidation products of a portion of the oleic acid, and not present originally. If these amounts be converted to oleic acid, the total oleic acid thus found agrees with that calculated from the iodine value of the original fat.

In the literature several references are found relating to the presence in butter fat of traces of acids other than those tabulated here. If such exist, they will probably be found amongst the higher fractions, the investigation of which we hope will form the subject of a future communication. In the

meantime, however, we intend to apply the method now described for a comparative study of fats obtained from different sources, and of milk fats produced under different conditions.

Experimental.

In the following pages full details are given of the method employed for the analysis of a sample of butter fat prepared from butter purchased at a local dairy. The general procedure followed was the same for the other materials analysed for which, therefore, only a condensed account is given.

I. *Analysis of Cow's Butter Fat.*

Preparation of the fat for analysis.

The butter was placed in a beaker, and melted by warming at a temperature not above 50°. As soon as the water and curd had separated, leaving the upper layer of fat perfectly clear, it was decanted through a folded filter paper, which had previously been thoroughly dried in the steam oven. The filtered fat was then ready for analysis. If not required at once it should be preserved in the cold away from light and air. In melting the butter a high temperature must be avoided, but at the same time the duration of heating should be reduced to a minimum.

Preparation of the methyl esters from butter fat.

From preliminary experiments it was found that Haller's [1906, 1907, 1908] acid method of esterification was preferable to that employed by Bull [1900, 1906], but to render the process quantitative, several precautions must be taken. These will be mentioned in the account which follows.

136·11 g. clarified fat were weighed off accurately into a round-bottomed litre flask and mixed with 214 cc. absolute methyl alcohol containing 2·5 % hydrogen chloride, and 323 cc. pure dry ether. The flask used was fitted to a fairly long double-surface condenser by means of a ground-glass joint, and the condenser carried a delivery tube, which was bent twice at right angles, and led into a series of U-tubes, surrounded by a freezing mixture of ice and salt. The esterification mixture was then heated for twelve hours on a water-bath at a temperature just sufficient to keep the liquid refluxing gently. After allowing to cool thoroughly, the U-tubes were detached, and the small quantity of liquid (10 to 15 cc.), which had collected, transferred to a litre graduated flask (*A*). The tubes were washed out with a little pure dry ether, and the washings also added to flask (*A*). The liquid, which had collected

in these tubes, was mainly ether, but at the same time an appreciable quantity of methyl ester (methyl butyrate) was always found to be present. Consequently, if this precaution was not taken, loss of this ester resulted.

To the ether-alcoholic solution of esters in the reaction flask, slightly more than the calculated amount of barium carbonate was added to remove the free hydrogen chloride. Though the mixture was frequently shaken, neutralisation proceeded slowly, but finally all the mineral acid was removed as indicated by Congo paper. The flask was then fitted with a five-pear still head and the ether and methyl alcohol distilled off, using a long double-surface condenser, and collecting the distillate in a flask cooled in a freezing mixture. The distillate, which contained in addition to ether and alcohol the greater part of the butyric ester, was then transferred to flask (*A*), the collection flask being washed out with ether, and the washings also added to (*A*). The residue, which consisted of methyl esters, glycerol, barium chloride, the excess of barium carbonate used, and the traces of solvents, was transferred by means of ether to a separating funnel and shaken with brine. This caused the ethereal solution of esters to separate out on top free from glycerol which was held in the salt solution. The process was repeated three times, and the separated ethereal solution then dried over anhydrous freshly ignited magnesium sulphate. After standing over night the magnesium sulphate was filtered off through a large Gooch crucible prepared in the usual way, and washed well with dry ether.

Fractional distillation of esters.

A 250 cc. Claisen distilling flask was fitted with a dropping funnel and thermometer, a portion of the dried ethereal solution of esters introduced through the funnel, and the ether distilled off carefully on the water-bath, the distillate being again collected in a cooled receiver, so as to avoid any loss of esters. As the ether evaporated, more solution was added by means of the tap-funnel until the whole had been introduced, and finally the flask and funnel were washed with pure dry ether. When all the ether had been distilled off, the receiver was removed, its contents transferred to flask (*A*), and in its place was fitted a "Perkin fractionation triangle." The tap-funnel was removed from the straight neck of the Claisen flask, which was now fitted with a cork, carrying a tube drawn out to a *very* fine capillary, and reaching to the bottom of the flask. The distillation of the esters was then carried out, at first at ordinary pressure, and then *in vacuo*, the distilling flask being heated by means of an asbestos air-bath. After the fraction boiling

about 140°/15 mm. had been collected the condenser was dispensed with, and the residual esters transferred with ether to a smaller Claisen flask, the side limb of which was connected directly with the receiver.

The first fraction that distilled over consisted mainly of ether, and consequently was added to the litre flask (*A*), which contained all the solvent alcohol and ether recovered from the process. The volume of this was now made up to the mark with pure alcohol (or ether) and the amount of methyl ester in solution determined as described below.

It was found that, as a rule, the fractionation must be repeated three or four times before a sufficient separation was effected. Appended are the details of the final (third) fractionation in the experiment described:

Fraction	Temperature °C.	Pressure mm.	Weight g.
1	65–130	atmospheric	1·6690
2	–150 53– 75	atmospheric 15	1·9662
3	–110	15	1·3396
4	–135	15	1·0886
5	–155	15	2·4600
6	–185	15	14·1852
7	–200 184–190	15 13	18·3872
8	–197	13	18·1542
9	–202	13	18·1230
10	–202	12	16·7630
11	–206	12	18·6558
12	–211	12	10·0762
13 (residue)	—	—	6·7827
	Total weight of fractions		129·6507

The temperatures quoted for the various fractions indicate the points at which the first and last drops contained in the respective fractions came over.

On comparison it will be seen that the boiling points given on p. 140 fall between these temperatures, and that it was about these points that the bulk of the fraction distilled. For example, in the above fractionation fraction 3 corresponds to (*d*) on p. 140, fraction 5 to (*f*), fractions 7 to 11 (inclusive) to (*h*). The collection of the last named in five fractions was necessitated simply by the relatively large volume of distillate obtained at this stage, collection-tubes of uniform size being used for general convenience.

Determination of methyl butyrate volatilised with solvents.

A 250 cc. conical flask was fitted with a cork carrying a dropping funnel and a delivery tube, the latter being connected with a condenser. 20 cc. of alcoholic KOH solution (roughly N/2) were transferred to the flask and

100 cc. of the ether-alcoholic solution from flask (*A*) placed in the funnel, the stem of which, drawn out to deliver small drops, reached almost to the surface of the potash solution. The flask was then heated on the water-bath, and the ether-alcoholic solution allowed to drop in slowly, so that the volume in the flask remained about the same. When all the solution had been added the distillate was transferred to the funnel, and the distillation repeated. All the ester in the original solution was thus hydrolysed, and after cooling the contents of the flask a few drops of phenolphthalein were added, and the excess of potash titrated with standard (roughly N/2) hydrochloric acid.

A blank experiment was carried out at the same time, using a mixture of 50 cc. pure ether and 50 cc. neutral alcohol in place of the ether-alcoholic solution of ester, and proceeding exactly as described above. From the difference between the two titres the amount of ester can be calculated if the reasonable assumption be made, that it consists solely of methyl butyrate. In all cases the mean of two closely agreeing determinations was used in calculating the results.

Appended are the details for the analysis here described:

	cc. HCl (N/1 × 0·497) required	
	a	*b*
Blank experiment	31·89	31·86
100 cc. ester solution	21·05	21·00
Difference	10·84	10·86
Mean titre	10·85 cc.	

Hence weight of methyl butyrate in solution (1000 cc.)

$$= 10{\cdot}85 \times 10 \times 0{\cdot}497 \times 0{\cdot}1021 \text{ g.}$$

$$= 5{\cdot}507 \text{ g.}$$

If this weight be now added to the 129·651 g. of esters obtained in the fractionation, a total of 135·158 g. of esters is arrived at as having been obtained from 136·11 g. of butter fat.

Examination of the individual fractions.

(1) *Iodine value.* The iodine value of each fraction was determined, using the method of Wijs. [Cf. Leathes 1910, pp. 67–69.]

The Wijs solution was always made up two or three days before being required for analysis, and before use its strength was determined by checking it against the thiosulphate solution, the latter being standardised by titration with weighed quantities of pure iodine.

From the iodine absorption of each fraction, the weight of methyl oleate present in it was then calculated, on the assumption that no other unsaturated compound was present.

The results obtained are summarised in the following table:

10 cc. Wijs reagent (= 21·92 cc. thiosulphate) were used in each case.

1 cc. thio. = 0·01257 g. iodine.

Fraction	Weight taken	Thio. used	Δ	Iodine value	Weight of methyl oleate in fraction
	g.	cc.	cc.	g.	g.
1	0·1775	21·76	0·16	01·13	0·022
2	0·2224	21·56	0·36	02·03	0·047
3	0·2642	20·59	0·33	06·33	0·106
4	0·2805	19·10	2·82	12·63	0·160
5	0·3017	19·35	2·57	10·70	0·307
6	0·2797	18·63	3·29	14·78	2·443
7	0·2564	15·81	6·11	30·07	6·294
8	0·2982	13·65	8·27	34·85	7·375
9	0·3257	9·56	12·36	47·70	10·080
10	0·2929	8·26	13·66	58·61	11·450
11	0·2559	8·83	13·09	64·26	13·970
12	0·2721	7·32	14·60	67·43	7·912
13	0·2904	9·25	12·67	54·84	4·335
			Total weight of methyl oleate		64·501

(2) *The saponification value.* The usual method for determining the saponification value of a fat was followed, that is, a weighed portion of each fraction was heated with excess of approximately semi-normal alcoholic potash, and the excess then estimated by titration with standard (0·497 N/1) hydrochloric acid, using phenolphthalein as indicator.

As all the fractions, except the last, were perfectly colourless liquids, the solutions for titration were only faintly coloured, and hence it was possible to obtain a very sharp end-point, thus giving results of considerable accuracy. A blank experiment was always carried out at the same time, and under identical conditions, and the difference between the two titres thus obtained corresponded to the fatty acids contained in and liberated during the process from the weight of esters taken for analysis.

The actual saponification numbers of the several fractions were not calculated, as the real object of the experiment was the determination of the mean molecular weights of the *saturated esters* present in each fraction. This was arrived at in the following way.

Having found the iodine value of each fraction, the amount of methyl oleate (b)[1] contained in the weight of esters (a) taken for the analysis of each fraction can readily be calculated, and the number of cc. of the standard hydrochloric acid (b') equivalent to these amounts of methyl oleate can then be obtained. If these titres for methyl oleate (b') be subtracted from the titres found for the total esters (a'), titres corresponding to the amounts of

[1] The index letters refer to the first table on p. 152

saturated esters (c') are arrived at, and hence the mean molecular weights of the saturated esters in each fraction can be determined, as the weights of the saturated esters (c) are also known. The complete results are tabulated below.

Fraction	(a) Weight taken	(b) Weight of oleate	(c) Weight of saturated esters	(a') Titre	(b') Titre for oleate	(c') Titre for saturated esters	Mean molecular weight of saturated esters
	g.	g.	g.	cc.	cc.	cc.	
1	0·5237	0·0069	0·5168	6·21	0·04	6·17	108·55
2	0·6023	0·0143	0·5880	9·22	0·09	9·13	129·6
3	0·6520	0·0517	0·6003	8·11	0·35	7·76	155·7
4	0·6701	0·0987	0·5714	7·23	0·67	6·56	175·3
5	0·7225	0·0901	0·6324	6·97	0·61	6·36	200·1
6	1·6495	0·2841	1·3654	13·80	1·93	11·87	231·45
7	1·7611	0·6028	1·1583	13·35	4·09	9·26	251·65
8	1·6410	0·6669	0·9741	12·32	4·53	7·79	251·7
9	1·6894	0·9392	0·7502	12·32	6·39	5·93	254·4
10	1·7109	1·1685	0·5424	12·11	7·94	4·17	262·05
11	1·6915	1·2520	0·4395	11·88	8·51	3·37	262·55
12	1·6942	1·3110	0·3832	11·88	9·05	2·83	270·4
13	1·6895	1·0800	0·6095	11·30	7·34	3·96	309·7

Fraction	Total weight	Weight of methyl oleate	Weight of saturated esters
	g.	g.	g.
1	1·6690	0·022	1·647
2	1·9662	0·047	1·920
3	1·3396	0·106	1·233
4	1·0886	0·160	0·928
5	2·4600	0·307	2·153
6	14·1852	2·443	11·742
7	18·3872	6·294	12·093
8	18·1542	7·375	10·779
9	18·1230	10·080	8·043
10	16·7630	11·450	5·313
11	18·6558	13·970	4·686
12	10·0762	7·912	2·164
13	6·7827	4·335	2·448
Totals	129·6507	64·501	65·149

Weights of Individual Esters in each Fraction.

Fraction	Butyric	Caproic	Caprylic	Capric	Lauric	Myristic	Palmitic	Stearic	Dihydroxy-stearic	Oleic
	g.	g.	g.	g.	g.	g.	g.	g.	g.	g.
Volatile with solvents	5·507	—	—	—	—	—	—	—	—	—
1	1·194	0·453	—	—	—	—	—	—	—	0·022
2	0·270	1·650	—	—	—	—	—	—	—	0·047
3	—	0·090	1·143	—	—	—	—	—	—	0·106
4	—	—	0·320	0·608	—	—	—	—	—	0·160
5	—	—	—	0·993	1·160	—	—	—	—	0·307
6	—	—	—	—	4·072	7·670	—	—	—	2·443
7	—	—	—	—	—	7·640	4·453	—	—	6·294
8	—	—	—	—	—	6·774	4·005	—	—	7·375
9	—	—	—	—	—	4·382	3·661	—	—	10·080
10	—	—	—	—	—	1·389	3·924	—	—	11·450
11	—	—	—	—	—	1·149	3·537	—	—	13·970
12	—	—	—	—	—	—	2·130	0·034	—	7·912
13	—	—	—	—	—	—	—	1·493	0·955	4·335
Totals	6·971	2·193	1·463	1·601	5·232	29·004	21·710	1·527	0·955	64·501

Final Summary.

Acid	Weight of ester	Ester	Acid*
	g.	%	%
Butyric	6·971	5·16	4·45
Caproic	2·193	1·62	1·45
Caprylic	1·463	1·08	0·99
Capric	1·601	1·19	1·10
Lauric	5·232	3·80	3·55
Myristic	29·004	21·46	20·13
Palmitic	21·710	16·08	15·24
Stearic	1·527	1·13	1·08
Dihydroxystearic	0·955	0·71	0·68
Oleic	64·501	47·74	45·47

* Strictly speaking the numbers given represent the weights of the respective acids obtainable from 100 g. of the mixed methyl esters, but must approximate very closely to the percentages in the actual fat.

II. *Analysis of Artificial Mixture of Fatty Acids.*

Details of the final distillation, and the iodine value of each fraction have already been given (p. 142).

Estimation of methyl butyrate:

Total ester required for saponification KOH = (86·9 × 0·497) cc. N/1 HCl.

Therefore total weight of methyl butyrate = 86·9 × 0·497 × 0·1021 = 4·41 g.

Total weight of esters obtained = 89·53 + 4·41 = 93·94 g.

" " " calculated = 93·97 g.

Analysis of Fractions.

Fraction	Iodine value	Mean molecular weight of saturated esters	Total weight of fraction	Weight of methyl oleate in fraction	Weight of saturated esters in fraction
	g.		g.	g.	g.
1	1·87	102·0	0·29	0·004	0·286
2	2·52	103·55	0·33	0·007	0·323
3	1·73	129·3	1·27	0·025	1·245
4	4·32	136·9	0·90	0·045	0·855
5	5·82	160·2	1·18	0·080	1·100
6	2·32	190·4	1·70	0·046	1·654
7	2·84	214·9	2·90	0·096	2·804
8	3·63	238·2	8·61	0·365	8·245
9	8·25	243·3	14·12	1·361	12·759
10	39·12	260·0	14·77	6·747	8·023
11	57·51	270·9	16·72	11·230	5·490
12	68·00	270·9	18·14	14·400	3·740
13	72·61	297·1	6·80	5·766	1·034
14	71·50	319·9	1·80	1·503	0·297
		Totals	89·53	41·675	47·855

Weights of Individual Esters in each Fraction.

Fraction	Butyric	Caproic	Caprylic	Capric	Lauric	Myristic	Palmitic	Stearic	Dihydroxy-stearic	Oleic
	g.	g.	g.	g.	g.	g.	g.	g.	g.	g.
Volatile with solvents	4·410	—	—	—	—	—	—	—	—	—
1	0·286	—	—	—	—	—	—	—	—	0·004
2	0·302	0·021	—	—	—	—	—	—	—	0·007
3	0·028	1·217	—	—	—	—	—	—	—	0·025
4	—	0·616	0·239	—	—	—	—	—	—	0·045
5	—	—	1·001	0·099	—	—	—	—	—	0·080
6	—	—	—	1·368	0·286	—	—	—	—	0·046
7	—	—	—	—	2·703	0·101	—	—	—	0·096
8	—	—	—	—	1·005	7·240	—	—	—	0·365
9	—	—	—	—	—	12·100	0·659	—	—	1·361
10	—	—	—	—	—	2·667	5·356	—	—	6·747
11	—	—	—	—	—	—	8·905	0·325	—	11·230
12	—	—	—	—	—	—			—	14·400
13	—	—	—	—	—	—	0·030	1·004	—	5·766
14	—	—	—	—	—	—	—	0·087	0·210	1·503
Totals	5·026	1·854	1·240	1·467	3·994	22·108	14·950	1·416	0·210	41·675
									equal to	0·188
										41·863
Theory	5·030	1·893	1·207	1·406	4·066	21·760	15·299	1·322	—	41·986

III. *Analysis of Butter Fat from "First Runnings" of Milk. July* 27–31, 1914.

The sample was prepared from the mixed "first runnings" (*i.e.* first drawn milk) of several cows, the milk drawn representing about one-quarter of the total yield of the cows. The whole of the milk thus obtained was passed through a cream separator, the cream churned without delay, and the butter thus obtained used for the analysis.

The fat gave a Reichert-Wollny number of 28·37 and an iodine value of 45·65. The latter corresponds to 50·68 % of oleic acid in the fat.

Complete analysis:

Weight of fat taken 157·54 g.
Volume of methyl alcohol containing 2·5 % HCl 248 cc.
Volume of ether 375 cc.

Final (3rd) Fractionation.

Fractions from 2nd fractionation introduced	Fraction	Temperature °C.	Pressure mm.	Weight g.
1, 2 →	1	65–130	760	2·3970
3 →	2	–150	760	1·4453
4 →		53– 78	15	
	3	–110	16	1·3808
5, 6, 7 →	4	–133	14	1·6900
	5	–160	14	2·6844
	6	–180	13	10·7606
8, 9 →	7	–188	13	13·3677
	8	–196	14	14·3442
	9	–205	14	18·3769
10, 11 →	10	200–203·5	13	16·5646
	11	–205·5	13	15·1847
12, 13 →	12	–208	13	16·1208
	13	206·5–210	13	18·9202
	14	–280	13	14·7388
			Total weight	147·976

Estimation of methyl butyrate:

Total ester required for saponification KOH = 123·5 × 0·523 cc. N/1 HCl.
Therefore total weight of methyl butyrate = 123·5 × 0·523 × 0·1021 = 6·59 g.
Total weight of esters obtained = 147·976 + 6·59 = 154·566 g.

Analysis of Fractions.

Fraction	Iodine value g.	Mean molecular weight of saturated esters	Weight of methyl oleate in fraction g.	Weight of saturated esters in fraction g.
1	0·24	117·8	0·007	2·390
2	1·08	125·15	0·018	1·427
3	3·28	141·6	0·053	1·328
4	8·06	166·7	0·159	1·530
5	12·73	182·0	0·408	2·276
6	13·89	221·0	1·746	9·015
7	19·20	236·45	2·997	10·370
8	28·06	257·2	4·701	9·643
9	37·59	264·35	8·067	10·310
10	48·01	271·6	9·289	7·276
11	55·95	272·2	9·920	5·265
12	60·34	286·5	11·360	4·761
13	68·03	289·1	15·040	3·880
14	65·26	302·2	11·235	3·504
		Totals	75·000	72·975

Weights of Individual Esters in each Fraction.

Fraction	Butyric	Caproic	Caprylic	Capric	Lauric	Myristic	Palmitic	Stearic	Dihydroxy-stearic	Oleic
	g.	g.	g.	g.	g.	g.	g.	g.	g.	g.
Volatile with solvents	6·590	—	—	—	—	—	—	—	—	—
1	0·909	1·481	—	—	—	—	—	—	—	0·007
2	0·206	1·221	—	—	—	—	—	—	—	0·018
3	—	0·721	0·607	—	—	—	—	—	—	0·053
4	—	—	1·001	0·530	—	—	—	—	—	0·159
5	—	—	0·283	1·993	—	—	—	—	—	0·408
6	—	—	—	—	6·546	2·469	—	—	—	1·746
7	—	—	—	—	1·860	8·510	—	—	—	2·997
8	—	—	—	—	—	4·148	5·495	—	—	4·701
9	—	—	—	—	—	1·904	8·406	—	—	8·067
10	—	—	—	—	—	—	6·823	0·453	—	9·289
11	—	—	—	—	—	—	4·812	0·453	—	9·920
12	—	—	—	—	—	—	1·843	2·918	—	11·360
13	—	—	—	—	—	—	1·100	2·780	—	15·040
14	—	—	—	—	—	—	—	3·011	0·492	11·235
Totals	7·705	3·423	1·891	2·523	8·406	17·031	28·479	9·615	0·492	75·000

Summary.

Ester	Wt. isolated	Ester	Acid
	g.	%	%
Butyric ...	7·705	4·99	4·30
Caproic	3·423	2·22	1·98
Caprylic ...	1·891	1·22	1·11
Capric	2·523	1·63	1·51
Lauric	8·406	5·44	5·08
Myristic ...	17·031	11·02	10·38
Palmitic ...	28·479	18·43	17·47
Stearic	9·615	6·22	5·93
Dihydroxystearic	0·492	0·32	0·30
Oleic	75·000	48·55	46·49
Total weight	154·565		

IV. *Analysis of Butter Fat from "Strippings" (July 27–31, 1914).*

The sample was prepared from the mixed "strippings" (last-drawn milk) of the same cows, and same milkings as furnished the sample of "first runnings," and was dealt with in precisely the same way.

The fat gave a Reichert-Wollny number of 29·38, and an iodine value of 44·02, the latter corresponding to 48·88 % of oleic acid in the fat. The fat was thus slightly richer in total volatile acids and slightly poorer in oleic acid than the fat of the "first runnings."

Complete analysis:

Weight of fat taken 158·10 g.

Volume of methyl alcohol containing 2·5 % HCl 248 cc.

Volume of ether 375 cc.

Final (3rd) Fractionation.

Fractions from 2nd fractionation introduced	Fraction	Temperature ° C.	Pressure mm.	Weight g.
1*	1	65–130	760	0·7170
2*	2	–150 / 53– 77	760 / 17	1·5583
3*	3	–110	17	1·0808
4*	4	–130	17	1·7800
5*	5	–140	17	1·7644
6, 7, 8 →	6	–160	18	1·4912
	7	–185	18	15·1776
9, 10 →	8	189–193	18	12·0946
	9	–201	19	13·8648
11 →	10	199–203	17	13·3750
	11	–206	17	14·4274
12, 13, 14 →	12	–208	17	16·7288
	13	–213	17	14·0042
	14	211–213	17	17·1292
15 →	15	–220	17	18·0496
	16 (residue)	—	—	5·3868
			Total weight	148·6297

* Fractions 1–5 of second fractionation were not redistilled.

Estimation of methyl butyrate:

Total ester required for saponification KOH = (122·8 × 0·523) cc. N/1 HCl.

Therefore total weight of methyl butyrate = 122·8 × 0·523 × 0·1021 = 6·557 g.

Total weight of esters obtained = 148·630 + 6·557 = 155·187 g.

Analysis of Fractions.

Fraction	Iodine value	Mean molecular weight of saturated esters	Weight of methyl oleate in fraction	Weight of saturated esters in fraction
	g.		g.	g.
1	0·15	112·9	0·001	0·716
2	0·55	122·6	0·010	1·548
3	1·63	135·0	0·021	1·060
4	4·90	153·9	0·102	1·678
5	12·16	172·9	0·250	1·514
6	11·65	196·8	0·203	1·288
7	11·41	223·8	2·022	13·156
8	19·16	235·7	2·704	9·390
9	24·64	246·1	3·990	9·875
10	30·33	251·9	4·848	8·527
11	40·94	252·35	6·898	7·529
12	56·74	268·7	11·090	5·639
13	63·73	269·0	10·420	3·584
14	68·21	285·4	13·650	3·479
15	68·91	288·1	14·620	3·430
16	66·86	304·8	4·208	1·179
		Totals	75·037	73·592

Weights of Individual Esters in each Fraction.

Fraction	Butyric	Caproic	Caprylic	Capric	Lauric	Myristic	Palmitic	Stearic	Dihydroxy-stearic	Oleic
	g.	g.	g.	g.	g.	g.	g.	g.	g.	g.
Volatile with solvents	6·557	—	—	—	—	—	—	—	—	—
1	0·398	0·318	—	—	—	—	—	—	—	0·001
2	0·343	1·205	—	—	—	—	—	—	—	0·010
3	—	0·844	0·216	—	—	—	—	—	—	0·021
4	—	0·215	1·463	—	—	—	—	—	—	0·102
5	—	—	0·648	0·866	—	—	—	—	—	0·250
6	—	—	—	0·748	0·540	—	—	—	—	0·203
7	—	—	—	—	8·175	4·981	—	—	—	2·022
8	—	—	—	—	1·918	7·472	—	—	—	2·704
9	—	—	—	—	—	8·287	1·588	—	—	3·990
10	—	—	—	—	—	5·295	3·232	—	—	4·848
11	—	—	—	—	—	4·550	2·979	—	—	6·898
12	—	—	—	—	—	0·236	5·403	—	—	11·090
13	—	—	—	—	—	0·115	3·469	—	—	10·420
14	—	—	—	—	—	—	1·481	1·998	—	13·650
15	—	—	—	—	—	—	1·136	2·294	—	14·620
16	—	—	—	—	—	—	—	0·908	0·271	4·208
Totals	7·298	2·582	2·327	1·614	10·633	30·936	19·288	5·200	0·271	75·037

Summary.

Ester	Wt. isolated	Ester	Acid
	g.	%	%
Butyric ...	7·298	4·70	4·06
Caproic	2·582	1·66	1·48
Caprylic ...	2·327	1·50	1·37
Capric	1·614	1·04	0·96
Lauric	10·633	6·85	6·40
Myristic ...	30·936	19·93	18·78
Palmitic ...	19·288	12·43	11·78
Stearic	5·200	3·35	3·19
Dihydroxystearic	0·271	0·17	0·16
Oleic	75·037	48·36	41·31
Total weight	155·186		

V. *Analysis of Butter Fat from Ewe's milk.*

The sample was prepared from the mixed milk of several ewes of the flock at the Manor Farm, Garforth, in the spring of 1914.

The fat was separated by churning, and dealt with in precisely the same way as the samples of cow's butter fat.

The fat gave a Reichert-Wollny number of 29·41, and an iodine value of 41·41.

Complete analysis:

Weight of fat taken 162·16 g.

Volume of methyl alcohol containing 2·5 % HCl 280 cc.

Volume of ether 250 cc.

Final (4th) Fractionation.

Fraction	Temperature ° C.	Pressure mm.	Weight g.
1	–150	760	0·65
2	–160	760	2·30
3	55 – 77	16	1·25
4	–110	15	3·46
5	–130	15	8·15
6	–143	15	1·31
7	–155	15	4·97
8	–185	14	10·88
9	–190	13	16·83
10	–196	12	15·46
11	–201	12	17·14
12	–204	12	19·08
13	–207	12	15·72
14	–210	12	18·16
15	–220	12	9·10
16	–300	12	4·81
		Total weight	149·27

Estimation of methyl butyrate:

Total ester required for saponification KOH = (248·35 × 0·523) cc. N/1 HCl.
Therefore total weight of methyl butyrate = 248·35 × 0·523 × 0·1021 = 12·26 g.
Total weight of esters obtained = 149·27 + 12·26 = 161·53 g.

Analysis of Fractions.

Fraction	Iodine value g.	Mean molecular weight of saturated esters	Weight of methyl oleate in fraction g.	Weight of saturated esters in fraction g.
1	0·075	125·7	0·001	0·649
2	0·365	145·7	·010	2·290
3	0·55	146·8	·008	1·242
4	1·525	159·8	·062	3·398
5	3·34	177·6	·318	7·832
6	3·72	187·8	·057	1·253
7	3·70	206·9	·214	4·756
8	8·64	228·5	1·098	9·782
9	17·19	245·7	3·378	13·452
10	28·30	254·3	5·110	10·350
11	44·59	269·2	8·926	8·214
12	53·55	273·9	11·935	7·145
13	64·73	285·7	11·882	3·838
14	68·54	288·1	14·456	3·704
15	70·61	298·8	7·503	1·597
16	69·01	316·5	3·876	0·934
		Totals	68·834	80·436

Weights of Individual Esters in each Fraction.

Fraction	Butyric	Caproic	Caprylic	Capric	Laurie	Myristic	Palmitic	Stearic	Dihydroxy-stearic	Oleic
	g.	g.	g.	g.	g.	g.	g	g.	g.	g.
Volatile with solvents	12·260	—	—	—	—	—	—	—	—	—
1	0·083	0·566	—	—	—	—	—	—	—	0·001
2	—	0·909	1·381	—	—	—	—	—	—	0·010
3	—	0·446	0·796	—	—	—	—	—	—	0·008
4	—	—	3·146	0·252	—	—	—	—	—	0·062
5	—	—	2·093	5·739	—	—	—	—	—	0·318
6	—	—	—	1·161	0·092	—	—	—	—	0·057
7	—	—	—	1·084	3·672	—	—	—	—	0·214
8	—	—	—	—	4·416	5·366	—	—	—	1·098
9	—	—	—	—	—	11·522	1·930	—	—	3·378
10	—	—	—	—	—	5·522	4·828	—	—	5·110
11	—	—	—	—	—	0·211	8·003	—	—	8·926
12	—	—	—	—	—	—	6·062	1·083	—	11·935
13	—	—	—	—	—	—	1·593	2·245	—	11·882
14	—	—	—	—	—	—	1·227	2·477	—	14·456
15	—	—	—	—	—	—	—	1·554	0·043	7·503
16	—	—	—	—	—	—	—	0·371	0·563	3·876
Totals	12·343	1·921	7·416	8·236	8·180	22·621	23·643	7·730	0·606	68·834

Summary.

Ester	Wt. isolated g.	Ester %	Acid %
Butyric ...	12·343	7·64	6·60
Caproic	1·921	1·19	1·06
Caprylic ...	7·416	4·59	4·18
Capric	8·236	5·10	4·72
Lauric	8·180	5·06	4·73
Myristic ...	22·621	14·01	13·20
Palmitic ...	23·643	14·64	13·87
Stearic	7·730	4·79	4·56
Dihydroxystearic	0·606	0·38	0·36
Oleic	68·834	42·63	40·61
Total weight	161·530		

VI. *Analysis of Butter Fat from Ewe's milk.*

Repetition of V with a further portion of same sample.

Complete analysis:

Weight of fat taken 164·35 g.

Volume of methyl alcohol containing 2·5 % HCl 273 cc.

Volume of ether 250 cc.

Final Fractionation.

Fraction	Temperature °C.	Pressure mm.	Weight g.
1	−140	760	0·89
2	−155 / 55 − 77	760 / 12	2·82
3	− 80	12	3·72
4	−110	11	5·65
5	−140	11	7·12
6	−155	11	4·93
7	−185	11	17·56
8	184 −190	11	17·63
9	−192	11	17·54
10	−195	11	18·33
11	−199	11	17·71
12	−201	11	18·32
13	−215	11	12·45
14	Residue	—	6·94
	Total weight		151·61

Estimation of methyl butyrate:

Total ester required for saponification KOH = (227·9 × 0·523) cc. N/1 HCl.
Therefore total weight of methyl butyrate = 227·9 × 0·523 × 0·1021 = 12·17 g.
Total weight of esters obtained = 151·61 + 12·17 = 163·78 g.

Analysis of Fractions.

Fraction	Iodine value g.	Mean molecular weight of saturated esters	Weight of methyl oleate in fraction g.	Weight of saturated esters in fraction g.
1	0·01	124·9	0·001	0·89
2	1·62	145·0	0·053	2·77
3	3·00	154·0	0·131	3·59
4	3·74	169·7	0·247	5·40
5	4·42	190·2	0·368	6·75
6	6·50	210·4	0·374	4·56
7	11·40	235·9	2·337	15·22
8	25·09	247·1	5·166	12·46
9	38·97	261·3	7·981	9·56
10	48·01	271·0	10·280	8·05
11	61·70	277·0	12·760	4·95
12	67·63	286·6	14·470	3·85
13	70·18	299·1	10·200	2·25
14	69·53	303·3	5·636	1·30
		Totals	70·004	81·60

Weights of Individual Esters in each Fraction.

Fraction	Butyric	Caproic	Caprylic	Capric	Lauric	Myristic	Palmitic	Stearic	Dihydroxy-stearic	Oleic
	g.	g.	g.	g.	g.	g.	g.	g.	g.	g.
Volatile with solvents	12·170	—	—	—	—	—	—	—	—	—
1	0·135	0·755	—	—	—	—	—	—	—	0·001
2	—	1·167	1·603	—	—	—	—	—	—	0·053
3	—	0·449	3·141	—	—	—	—	—	—	0·131
4	—	—	2·929	2·471	—	—	—	—	—	0·247
5	—	—	—	5·610	1·140	—	—	—	—	0·368
6	—	—	—	0·518	4·042	—	—	—	—	0·374
7	—	—	—	—	3·007	12·213	—	—	—	2·337
8	—	—	—	—	—	9·977	2·483	—	—	5·166
9	—	—	—	—	—	2·751	6·809	—	—	7·981
10	—	—	—	—	—	—	7·734	0·316	—	10·280
11	—	—	—	—	—	—	3·618	1·332	—	12·760
12	—	—	—	—	—	—	1·477	2·373	—	14·470
13	—	—	—	—	—	—	—	2·168	0·082	10·200
14	—	—	—	—	—	—	—	1·066	0·234	5·636
Totals	12·305	2·371	7·673	8·599	8·189	24·941	22·121	7·255	0·316	70·004

Summary.

Ester	Wt. isolated	Ester	Acid
	g.	%	%
Butyric ...	12·305	7·51	6·48
Caproic	2·371	1·45	1·29
Caprylic ...	7·673	4·69	4·27
Capric	8·599	5·25	4·86
Lauric	8·189	5·00	4·67
Myristic ...	24·941	15·23	14·28
Palmitic ...	22·121	13·51	12·81
Stearic	7·255	4·43	4·22
Dihydroxystearic	0·316	0·19	0·18
Oleic	70·004	42·75	40·72
Total weight	163·774		

REFERENCES

Bell (1890). *Analysis and Adulteration of Foods*, **2**.
Blyth (1903). *Foods, their Composition and Analysis* (5th Edit.).
Browne (1899). *J. Amer. Chem. Soc.* **21**, 807.
Bull (1900). *Chem. Zeit.* **146**, 259.
—— (1906). *Ber.* **39**, 3570.
Caldwell and Hurtley (1909). *J. Chem. Soc.* **95**, 853.
Duclaux (1886). *Compt. rend.* **102**, 1022.
Fleischmann and Warmbold (1907). *Zeitsch. Biologie*, N.F. **32**, 375.
Haller (1906). *Compt. rend.* **143**, 657, 803.
—— (1907). *Compt. rend.* **144**, 462.
—— (1908). *Compt. rend.* **146**, 259.
Hehner and Mitchell (1896). *Analyst*, **21**, 320.
Holland, Reed and Buckley (1916). *J. Agric. Research*, **6**, 101.
Jensen (1905, 1). *Zeitsch. Nahr. Genussm.* **10**, 265.
—— (1905, 2). *Analyst*, **30**, 397.
Koefoed (1891). *Bull. acad. roy. danoise.* (*J. Chem. Soc. Abs.* 1892, **62**, 1113.)
—— (1892). *Zentralb. Agrik. Chem.* **41**, 202.
Leathes (1910). *The Fats* (Monographs on Biochemistry).
Lewkowitsch (1913). *Analysis of Oils, Fats and Waxes* (5th Edit.) **1**.
Meyer and Eckert (1910). *Monatsh.* 1232.
Molt, quoted by König (1907). *Chemie der Nahrungs u. Genussm.* (2nd Edit.).
Richmond (1914). *Dairy Chemistry* (2nd Edit.).
Siegfeld (1907). *Milch. Zentr.* **3**. 288.
Smedley (1912). *Biochem. J.* **6**, 451.

XVI. THE OXIDATION OF THE ALKALI BUTYRATES BY HYDROGEN PEROXIDE WITH THE PRODUCTION OF SUCCINIC ACID.

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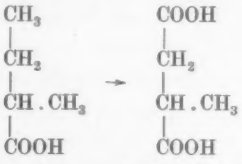
The oxidation of butyric acid in the form of its ammonium salt has been studied by Dakin [1908]. He slowly distilled a solution of ammonium butyrate containing a slight excess of ammonia with two molecular proportions of 3 % hydrogen peroxide. To identify an unstable intermediate product—acetoacetic acid—he allowed ammonium butyrate and hydrogen peroxide to act upon each other at 37°. Dakin supposes that the attack on the fatty acid occurs both at the α- and β-carbon atoms, the main attack being at the latter. The attack at the α-carbon atom will lead to the production of propionic aldehyde, propionic acid, acetaldehyde, acetic and formic acids: the attack at the β-carbon atom will yield acetoacetic acid, which will readily break up into acetone and carbon dioxide. Dakin was able to prove the formation of all the substances mentioned, and he claims that the yield of acetone is as high as 50 % of that theoretically possible.

In a paper recently published by Hurtley [1916] the theory of the oxidation of the fatty acids by attack at the β-carbon atom has been subjected to some criticism on the ground of its inadequacy. It was there suggested that the oxidative attack occurred simultaneously at many points in the long chains of carbon atoms which are present in all the common higher fatty acids and in particular that the attack might occur at the terminal methyl group. This is the more likely because Dakin and Herter [1907] have shown that when hydrogen peroxide attacks the benzene ring it does not show the discrimination which is shown by the usual substituting agents: thus, when it attacks benzoic acid it yields a mixture of all three hydroxybenzoic acids. Direct evidence that oxidation of fatty acids occurs at the methyl group in the animal body is not available. Only one piece of evidence, as far as the authors know, exists showing that it occurs at the methyl group when a fatty acid is oxidised by hydrogen peroxide, and this is furnished by Raper

[1914], who found that, when α-methylbutyric acid is oxidised by hydrogen peroxide, methylsuccinic acid is one of the products of the oxidation.



This surely indicates attack at the terminal methyl group. We have therefore taken up the question of the oxidation of the fatty acids by hydrogen peroxide and starting with butyric acid we have obtained results which lead us to suppose that the methyl group is a principal point of attack in this kind of oxidation and also encourage us to think that it will be worth while to study other fatty acids from this point of view. At first, following Dakin, we used the ammonium salt of butyric acid, but although the oxidation of this salt appears to take place more quickly than that of the sodium or potassium salts we have discarded it and now work with the sodium salt only, because:

(1) The ammonium salt gave rather strongly coloured products.

(2) It always left the product acid at the end of the reaction.

(3) In the body it is more probable that the sodium or potassium salts and not the ammonium salts are those that are oxidised.

(4) On submitting a liquid containing ammonium butyrate to steam distillation, both ammonia and butyric acid pass over with the steam.

To show this we distilled 200 cc. of a solution of ammonium butyrate containing 8·8 g. of butyric acid in a current of steam at such a rate that 50 cc. collected in about four minutes, collecting six fractions of 500 cc. each. In the table of results the first column gives the butyric acid in terms of normal sodium hydroxide: 50 cc. of the distillate were treated with a measured excess of normal sodium hydroxide, boiled till all ammonia was expelled and titrated with normal acid and phenolphthalein. These results are a little high. The second column gives the ammonia also in terms of normal sodium hydroxide: 50 cc. of the distillate were treated as in a Kjeldahl determination. This column gives the ammonia exactly.

Fraction	Butyric acid as cc. N NaOH	Ammonia as cc. N NaOH
1	32	36
2	23	19
3	14	12
4	10	8
5	8	6
6	8	5
Residue	19	14

It will be seen that the first distillate is alkaline and all the rest and the residue are acid in reaction. We wished to be able to distil our products without fear of obtaining ammonia in the distillate.

Our method of working is different from that of Dakin. After many preliminary experiments we adopted the following procedure: 1 g.-molecular proportion of sodium butyrate, equivalent to 4·4 g. of butyric acid, was dissolved in water and 3, 6, or 12 g.-molecular proportions of hydrogen peroxide (20 volume H_2O_2 for the first two and 40 volume for the third) were added and the mixture made up to 190 cc. in all cases. In most of our experiments the mixture was heated at 65° in a flask with an efficient reflux condenser, until all, or all but a trace, of the hydrogen peroxide had disappeared. Temperature, as would be expected, has an exceedingly marked effect on the duration of the reaction: thus with 3 g.-molecules of peroxide at 37° the products are practically the same as at 65°, but the times required are 168 and 66 hours respectively. At 100° the reaction appears to be complete in four hours, but we observed that at this temperature much oxygen was given off and the product was much discoloured. At the end of an experiment with three atoms of oxygen the product is neutral: with six atoms of oxygen the reaction is acid: with twelve atoms of oxygen the reaction is again neutral, or slightly alkaline.

At the end of an oxidation the solution is neutralised, if it is not neutral. In one part aldehydes plus acetone are determined (*a*) directly by addition of standard iodine and caustic soda and titration of excess of iodine by standard thiosulphate in the usual way; (*b*) by distilling a part acidified with acetic acid exactly as in the Messinger-Huppert process. The other part is evaporated to dryness on the water-bath: acidified with a slight excess of normal sulphuric acid, and distilled in steam until all volatile acids are expelled: this usually requires 3000 cc. of distillate. This distillate is titrated and called volatile acids. The residue from this steam distillation is then extracted for 72 hours with ether in a continuous apparatus such as that described by one of us [Hurtley, 1916; see also Plimmer, 1915, p. 598]. After distilling off the ether the liquid is evaporated to dryness in a weighed platinum dish, the solid residue is dried at 100° and weighed. We call this weight the yield of crude acid. It is practically all succinic acid; the colour is usually slightly brown and it has a smell recalling that of pyruvic acid. A small portion of it is very sparingly soluble in water. To purify and identify the succinic acid it was redissolved in water, boiled with animal charcoal, filtered and crystallised. The crystals obtained were still slightly yellow

and were not improved by repeating this process of purification nor by recrystallising from alcohol. They melted at 185°, the melting point of succinic acid, and the melting point was not changed on mixing with succinic acid (Nos. 1, 2 and 3).

The acid was finally obtained pure by recrystallisation from pure concentrated nitric acid and then from water (No. 4).

Analysis:

				C	H
1.	0·2513 g.	0·3875 g. CO_2	0·1170 g. H_2O	42·06	5·17
2.	0·2408 g.	0·3713 g. „	0·1092 g. „	42·06	5·05
3.	0·2264 g.	0·3484 g. „	0·1059 g. „	41·97	5·19
4.	0·2345 g.	0·3502 g. „	0·1070 g. „	40·72	5·07
Calculated for succinic acid ...		... ...		40·67	5·09

The acid crystallises like succinic acid: in neutral solution it gives a buff coloured precipitate with ferric chloride. Of this acid 0·1672 g. required 14·4 cc. N/5 NaOH which corresponds to a molecular weight of 116: theory 118.

One g. of the acid was digested at 60° with 3–4 g. of acetyl chloride for three hours; on cooling it deposited beautiful crystals of succinic anhydride which melted at 119°–120°.

It is inconceivable that succinic acid is produced from butyric acid without the formation of intermediate products, and we have some evidence of the presence of such substances. The succinic acid before crystallisation from nitric acid gave high figures for the percentage of carbon and hydrogen; the acid also gave Schiff's test for aldehydes, and it reduced ammoniacal silver. All these observations would be explained by assuming that before crystallisation from nitric acid the acid contained a small amount of the half aldehyde of succinic acid. The following table will give some idea of the yield of succinic acid, of aldehydes and acetone taken together, and of carbon dioxide. In order to be able to calculate a yield for the succinic acid it is necessary to form an idea of the butyric acid left over and also of that part of it which has been converted into other products than succinic acid. At the end of one of our experiments the solution will contain (*a*) unchanged sodium butyrate, (*b*) sodium salts of lower fatty acids, (*c*) aldehydes (acetic and propionic as Dakin [1908] has shown) and acetone, (*d*) sodium succinate. If after neutralisation the liquid is evaporated to expel aldehydes and acetone, then acidified and distilled in steam, the distillate should give on titration, expressing the result as butyric acid, the sum of the unchanged butyric acid

and the other volatile fatty acids. Of the fatty acids in the distillate other than butyric, every molecule of propionic must correspond to one molecule of butyric acid oxidised. It is unlikely that acetic acid would arise from acetoacetic acid in our experiments, but it might arise from the oxidation of acetone. Some acetic acid must also be formed from the oxidation of propionic acid, but in each of these cases one molecule of acetic acid would correspond to one molecule of butyric acid oxidised. In the case of formic acid it is true that this acid might be formed in different ways so that each molecule of formic acid in our distillate would not represent one molecule of butyric acid oxidised, but perhaps more nearly one-third of a molecule. By far the greatest part of our volatile fatty acid is unchanged butyric acid and certainly it contains only a little formic acid, for in working up 250 g. of sodium salts from the steam distillate we obtained only a little carbon monoxide on treatment with pure concentrated sulphuric acid. We may therefore take the difference between the initial butyric acid and the titration of the volatile acid, expressed as butyric acid, as a measure of the butyric acid which has been converted into aldehydes, acetone and succinic acid, so that if we calculate the yield of succinic acid only on this difference we shall almost certainly be underestimating it. In the table the yield of succinic acid is so estimated.

From the table it is seen that the yield of succinic acid is variable even when the conditions appear to be the same; the only explanation we can offer of this phenomenon is that in some cases there may have been a greater loss of oxygen than in others. Nevertheless in spite of these differences certain results appear perfectly clearly:

1. A yield of 50 % and over of succinic acid can be obtained by oxidising sodium butyrate in neutral solution by hydrogen peroxide at 65°. It is therefore established that under these conditions a fatty acid such as butyric can be and is attacked to a very great extent at the methyl end of the chain.

2. The yield is much diminished in less concentrated solutions. In experiment number 2 when the volume is 1000 cc. instead of 720 cc. the yield of succinic acid is only 20 %.

3. When the concentration of the oxidising agent is doubled the yield of succinic acid is increased with respect to the butyric acid taken, but it is practically the same with respect to butyric acid oxidised. More acid is oxidised and the time taken is nearly doubled.

4. When the peroxide is quadrupled a more marked change occurs in the yield of the several products and in the time required to use up the

peroxide. The reaction is now complete in 23 hours; the amount of butyric acid oxidised is double the amount oxidised with three atoms of oxygen; the yield of succinic acid is greatly reduced while that of the carbon dioxide is enormously increased. Perhaps it is a fair inference to draw that under these conditions the succinic acid is formed, but a large part of it is oxidised as it is produced.

Number of experiment	Time in hours	Weight of butyric acid taken in g.	Total volume of solution in cc.	Hydrogen peroxide in atoms of available oxygen	Difference between column 3 and volatile acids calculated as butyric acid in g.	Weight of crude succinic acid in g.	Yield per cent. of theory of crude succinic acid	CO_2 evolved in g.	Aldehyde plus acetone calculated as butyric acid in g.	Remarks
1	48	17·6	720	3	3·08	2·13	51·5	—	—	At 65°
2	48	17·6	1000	3	5·63	1·54	20·3	—	—	,,
3	66	4·4	190	3	1·14	0·48	31·6	0·36*	—	,,
4	66	4·4	190	3	1·07	0·43	30·1	0·40*	—	,,
5	66	4·4	190	3	1·41	0·47	25·1	—	0·65	,,
6	66	4·4	190	3	0·75	0·37	36·3	—	0·50	,,
7	72	4·4	190	3	1·10	0·50	33·6	0·37*	—	,,
8	72	4·4	190	3	1·02	0·46	33·6	—	0·64	,,
9	72	4·4	190	3	0·75	0·51	50·7	0·54*	—	,,
10	72	4·4	190	3	0·79	0·51	48·4	—	0·60	,,
11	72	4·4	190	3	0·79	0·52	49·2	0·44	—	,,
12	66	4·4	190	3	1·23	0·48	29·3	—	0·71	,,
13	72	4·4	190	3	1·41	0·52	27·2	0·37	—	,,
14	168	4·4	190	3	1·23	0·32	19·5	—	0·47	At 37°
15	72	17·6	720	6	6·69	3·18	35·5	—	—	At 65°
16	114	4·4	190	6	1·63	0·72	32·8	0·48*	—	,,
17	114	4·4	190	6	1·54	0·69	33·2	—	1·10	,,
18	114	4·4	190	6	1·50	0·77	38·0	—	—	,,
19	114	4·4	190	6	1·36	0·71	38·8	—	—	,,
20	114	4·4	190	6	1·32	0·67	37·8	0·88	—	,,
21	114	4·4	190	6	1·89	0·72	28·5	—	1·16	,,
22	114	4·4	190	6	1·72	0·75	32·5	1·12	—	,,
23	672	4·4	190	6	0·79	0·71	67·3	—	0·73	At 37°
24	22	4·4	190	12	2·46	0·56	16·8	2·59	—	At 65°
25	23	4·4	190	12	2·46	0·51	15·4	—	0·80	,,
26	23	4·4	190	12	2·46	0·50	15·2	2·72	—	,,

* Determined by passing the gas through baryta water and titrating.

A number of carbon dioxide determinations have been made, some by the use of baryta water and others by direct weighing; with care both methods give practically the same result. We do not find that our hydrogen peroxide contains more than a trace of carbon dioxide. Dakin found that his specimens contained a great deal. We have found however that the hydrogen peroxide acts upon rubber stoppers producing sulphur dioxide, which is absorbed by the potash used for absorbing the carbon dioxide—but this action is quite slow and only affects experiments which are continued for many hours.

In the last column but one are given some determinations of aldehyde plus acetone which are calculated to butyric acid. All the figures in this column were obtained by distilling an aliquot part of the oxidised product exactly as in a Messinger-Huppert acetone determination. We have also made some determinations by titrating after Messinger-Huppert without previous distillation. There was always a marked difference between the two results, a difference we attribute to the presence of less volatile aldehydes or aldehydo-acids.

We have also attempted to make separate determinations of aldehydes and acetone using Tollens' reagent for oxidation of the aldehydes. The following table gives some of our results. The amount of butyric acid used for the experiments and the volume of the solution are the same in all the experiments, namely 4·4 g. of the acid neutralised by sodium hydroxide, and 190 cc.

Hydrogen peroxide in atoms of available oxygen	Aldehydes + acetone expressed as g. of butyric acid		Acetone expressed as g. of butyric acid	Yield* % of theory
	By distillation	By direct titration		
3	0·76	1·02	0·206	20
3	0·73	1·06	0·407	39
6	0·94	1·46	0·457	32
6	0·73	1·43	0·290	20†
12	0·79	—	0·250	11
12	0·84	0·96	0·323	14
12	0·86	0·93	0·217	10

* The yields are calculated on the *average* value of the butyric acid oxidised in each class of experiment because the volatile acids were not determined in all these experiments.

† Experiment at 37°.

From this table it is seen that by our method of oxidation we cannot approach Dakin's yield of 50 % of acetone. We believe our highest figure of 39 % is probably too high. Yet we believe that our method of working is a nearer approach to the conditions which prevail in the animal body than Dakin's method of actual distillation.

Butyric acid in the form of its sodium salt may be broken down by oxidation in three ways by hydrogen peroxide, namely by attack at each of its carbon atoms except that one present as carboxyl. Of these three modes of attack that at the terminal carbon atom results in the production of succinic acid; that at the β-carbon atom results in the production primarily no doubt of acetoacetic acid which is broken down to acetone and carbon dioxide; that at the α-carbon atom probably yields propionic aldehyde, then the acid, and so on. Of these three we believe the

attack at the methyl group to be by far the most important. Butyric acid is easily oxidised in the body; of the intermediate products of its oxidation by hydrogen peroxide succinic acid is known to be easily oxidised in the body. Thus v. Longo [1878] took 13 g. of sodium succinate in two portions in 24 hours; no trace of the acid could be found in the urine. Marfori [1896] shows the same for animals. Batelli and Stern [1911] have shown that the acid is oxidised by nearly every tissue of the body. Acetoacetic acid on the contrary is difficult of oxidation in the body [see Hurtley, 1916, p. 380]. Propionic acid is known to be easily oxidised by the body. We believe therefore that in the body butyric acid is mainly attacked at the terminal carbon atom. If this is the case the same should be true of the higher fatty acids, and we propose to seek for evidence of this mode of attack in the case of some fatty acids higher in the series than butyric acid.

SUMMARY.

It is shown that a yield of succinic acid of over 50 % of the theoretical can be obtained by the oxidation of sodium butyrate by hydrogen peroxide.

REFERENCES.

Batelli and Stern (1911). *Biochem. Zeitsch.* **30**, 172.
Dakin (1908). *J. Biol. Chem.* **4**, 77.
Dakin and Herter (1907). *J. Biol. Chem.* **3**, 419.
Hurtley (1916). *Quart. J. Med.* **9**, 301.
Longo, v. (1878). *Zeitsch. physiol. Chem* **1**, 213.
Marfori (1896). *Ann. Chim. Farm.* **23**, 193. *Abstract in J. Chem. Soc.* **72**, ii. 419.
Plimmer (1915). *Practical Organic and Biochemistry* (Longmans, London).
Raper (1914). *Biochem. J.* **8**, 320.

XVII. THE ALLEGED ANTINEURITIC PROPERTIES OF α-HYDROXYPYRIDINE AND ADENINE.

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In the hope of obtaining a compound which would possess antineuritic properties Williams [1916] prepared and tested a series of substances. By adopting this line of research he hoped to throw some light on the chemical identity of the active principle which cures polyneuritis in birds and beri-beri in man. He confined himself to the study of pyridine derivatives mainly because the active fraction obtained from rice polishings is closely associated with nicotinic acid and under certain treatment gives off a pyridine-like odour, whilst the colour reactions given by antineuritic substances with phosphotungstic and phosphomolybdic acids respectively are also characteristic of the hydroxypyridines. He prepared and tested a large series of pyridine derivatives including nicotinic, cinchomeric, quinolinic, 6-hydroxynicotinic and citrazinic acids, glutazine, α-hydroxypyridine, 2 : 4 : 6-trihydroxypyridine (and its anhydride) and 2 : 3 : 4-trihydroxypyridine. The last three of the above Williams found to be definitely active but the activity disappeared after some time. As there was no evidence of decomposition, Williams ascribed the loss of the antineuritic properties to isomerisation. To substantiate his hypothesis he made a detailed study of one of the active substances—α-hydroxypyridine. He found that there were two crystalline modifications of this substance, needles and granules. By treating polyneuritic birds with the needle variety Williams managed to effect in every case a definite cure or improvement; the granules on the other hand were inactive. The absolute inactivity of the granular modification of α-hydroxypyridine induced him to put forward the further suggestion that the polyneuritic condition is brought about not by the lack of a particular isomer, since isomers are in equilibrium in solution and any biological preference for one

would disturb the equilibrium and thus effect the corresponding conversion, but by a want of a certain type of potential energy supplied by the process of isomerisation of certain substances. In a subsequent investigation Williams in collaboration with Seidell [1916] isolated from yeast an active substance which they considered to be identical with adenine and which lost its antineuritic activity on keeping. This loss of therapeutic potency the authors also ascribed to isomerisation.

If isomerisation is really responsible for the therapeutic activity of antineuritic principles it is of great scientific importance and interest and it was our object to study the matter in detail. We chose α-hydroxypyridine, with which Williams claims to have achieved some success, as our starting point. Although we could confirm most of the facts about the chemistry of that substance we were unable by its use to effect anything which in our opinion could be considered as a cure or even an improvement in the condition of the polyneuritic birds. We have taken great care in following Williams' method in the preparation of the active needles and no doubt exists in our minds that our preparations were identical with those described by Williams. We have also taken great pains in watching our birds and nothing of clinical importance could have missed our notice.

Experimental.

Like Williams we prepared the α-hydroxypyridine by distilling hydroxynicotinic acid according to the method of Pechmann and Baltzer [1891]. The latter was made from the methyl ester of coumalic acid by the method of Pechmann and Welsh [1884]. The coumalic acid was obtained from malic acid by the method described by Pechmann [1891]. Before distillation the hydroxynicotinic acid was decolorised with charcoal and purified by recrystallising it several times from hot 50 % acetic acid. It always gave the characteristic reactions and correct melting point. The α-hydroxypyridine obtained by distilling the hydroxynicotinic acid gave the correct nitrogen figures on determination by the Dumas method.

When hydroxynicotinic acid is distilled the α-hydroxypyridine comes over as syrup which on cooling crystallises in a mixture of needles and non-acicular crystals which Williams classified as granules. In Williams' opinion the amount of moisture present in the process of distillation is a determining factor as to the relative amounts of the two crystalline modifications formed. We found that by excluding moisture entirely needles only were formed.

We therefore heated the hydroxynicotinic acid and the distilling apparatus at 110° for several hours before distilling. The receiver was also fitted with a $CaCl_2$ tube in order to exclude all traces of moisture. From a recent publication of Williams [1917] which reached us only lately we find that he independently adopted a similar procedure and like ourselves was able to obtain needles only. On cooling, the needles crystallise out from the syrup and finally complete solidification occurs with formation of a dense mass. In parts of the apparatus where the layer of crystals is thin, well-defined needles are discernible even after complete cooling down. The solidified syrup did not show a definite melting point, but melted somewhere between 94°–98°. On keeping the open apparatus under ordinary laboratory atmospheric conditions a marked change could be observed in the needles in those parts of the distilling flask where their crystalline form was well defined after complete cooling. The needles after about 10 days broke up by transverse fissures and ultimately changed into non-acicular crystals of a granular form. On heating the cooled down syrup at a temperature of 120° for 2–3 hours and then allowing the molten mass to cool slowly in the absence of moisture dissolving it in hot dehydrated benzene and adding dehydrated ligroin to the cold solution a milky turbidity was produced and after a short time a deposit of acicular crystals was formed. Few, if any, granules were obtained by this precipitation, but a preponderance of granular crystals was obtained on concentrating the mother liquor. The needles thus obtained melted at 108°–110° (uncorrected), whilst the granules melted at 106°–108° (uncorrected). It is to be remarked that the configuration of the needles obtained by the benzene-ligroin recrystallisation differed in the different preparations obtained by that method. We did not however study the conditions governing these differential configurations. When the freshly distilled α-hydroxypyridine is dissolved in hot water and crystallised from it a mixture of needles and prisms is obtained. It is thus seen that there are at least two distinct crystalline modifications of α-hydroxypyridine, namely needles and non-acicular crystals. It will however be remembered that the melting points of the needles obtained by direct distillation of hydroxynicotinic acid and the needles obtained by the benzene-ligroin crystallisation method are not the same and therefore the two varieties of needles cannot be considered to be identical.

Polyneuritis was induced in our pigeons by forcible feeding with rice, the birds receiving daily 20 g. each morning and evening. The crops of the birds were examined daily and if any hardness was observed the feeding was suspended until the bird became normal again. The time taken by the

birds to develop polyneuritis varied from two to five weeks. All the injections were done intramuscularly in the breast and freshly prepared crops of crystals were always used unless otherwise mentioned. Following the practice of Williams the crystals were always dissolved in a convenient quantity of distilled water immediately before injection. The birds under treatment were kept under very close observation. Varying doses were tried. Needles obtained by direct distillation and needles from benzene and ligroin crystallisation as well as crystals obtained by crystallisation from hot water were tested. The following protocol describes eight experiments.

Expt. 1. 10 mg. of freshly distilled α-hydroxypyridine (needles) were dissolved in 2 cc. of distilled water and injected intramuscularly in pigeon 151 at 4 p.m. The bird showed at the time paralysis of the legs and retracted neck. At 8 p.m. its head was found to be markedly improved, and the condition of improvement was maintained at 11 p.m. Next morning the bird had a relapse. 20 mg. of needles obtained by distillation on the previous day were administered and as the bird showed no improvement a further 0·1 g. of the same substance was injected two hours later. The condition became worse and the bird died during the night.

Expt. 2. Pigeon 219 walked with great difficulty and was entirely unable to use its wings; head normal. It received 50 mg. of needles of freshly distilled α-hydroxypyridine at 12 noon. It was slightly worse at 11 p.m. the same evening, and worse again next morning. On the third day the bird showed a retracted neck. On the fourth day its condition was still worse and finally it was found dead on the morning of the fifth day.

Expt. 3. Pigeon 189 could not fly and walked with difficulty. It received 10 mg. of needles of freshly distilled α-hydroxypyridine at 12 noon. Next day there was no change in condition. On the third day the bird was decidedly worse. On the fourth day its condition was still worse, no retracted neck however was to be observed. It was found dead on the fifth morning.

Expt. 4. Pigeon 229 was paralysed and showed retracted neck. It received 50 mg. of needles obtained by recrystallising the α-hydroxypyridine from benzene with ligroin at 11.30 a.m. At 8 p.m. the same day its condition was worse and it died at 3 p.m. the following day.

Expt. 5. Pigeon 205 only showed incipient symptoms. It received 200 mg. of freshly distilled needles at 11.45 a.m. It became worse during the day and developed a retracted neck. Next day an improvement was observed but it became worse again in the course of the day and was found dead on the morning of the third day after treatment.

Expt. 6. Pigeon 230 showed paralysed limbs. It received 2 mg. of freshly distilled needles at 10 a.m. No change occurred in its condition during the day, but it became gradually but decidedly worse during the next three days and died on the fourth day.

Expt. 7. Pigeon 222 showed paralysed limbs. It received 50 mg. of α-hydroxypyridine recrystallised from hot water at 10.30 a.m. No change in condition occurred during that day. The next day the bird was worse and developed a retracted neck. It became gradually worse during the day and was found dead the next morning.

Expt. 8. Pigeon 149 showed paralysed limbs. It received 10 mg. of needles obtained by recrystallisation from benzene with ligroin at 6 p.m. No improvement was observed at 11 p.m. the same evening and the condition remained unchanged for some time. On the fourth day the bird was worse. 50 mg. of needles of the same crop were injected at 10.30 a.m. on that day. The bird died at 1 o'clock in the afternoon.

It is seen from the above experiments that no cure or improvement in the condition of the birds was effected by the treatment. Some birds lived several days after being treated, but they were types of cases, well known to those who work with polyneuritic birds, which would have behaved similarly if they had received no treatment at all. In many of the above experiments we started our treatment as soon as polyneuritic symptoms manifested themselves in order to give the drug a good chance to act. Some of these cases have turned out to be of the prolonged type where the onset is mild and gradual and lasts several days before terminating fatally, others have proved themselves to be of the intermittent type, in which the well-declared symptoms are strikingly relieved for a few hours before the second onset takes place; after the second attack the birds usually succumb. Expts. 2, 3, 6, and 8 are characteristic of the prolonged type. Expts. 1 and 5 represent the intermittent type. Special attention is to be paid to Expt. 5. That bird turned out to be of the intermittent type. It was treated when the symptoms were not more than of a premonitory character and the dose it received was very high, namely 200 mg. In spite of that, the first attack developed in the usual way and the bird succumbed as is common in cases of this type after the second onset within two days of the manifestation of the early symptoms. A special experiment showed that the injection of this amount of α-hydroxypyridine produced no toxic symptoms in a normal pigeon. In spite of the fact that we used both varieties of needles in various doses we are unable from our experiments

to attribute any antineuritic properties to the needle variety of α-hydroxypyridine.

Reference was made above to the work of Williams and Seidell [1916] in connection with the isomerisation of adenine. They obtained an active substance from autolysed yeast by adsorption which they considered to be adenine and which on keeping became inactive. The inactive adenine on being treated with sodium ethylate in a sealed tube at 100° for four hours, according to the observations of these workers became active. While we were engaged in repeating some of these experiments there appeared a paper by Voegtlin and White [1916] in which a variety of experiments with adenine, all of a negative nature, were described. We have therefore repeated only the crucial experiments of Williams and Seidell, and like Voegtlin and White. were unable to confirm their results.

We prepared our adenine from a sample of pure adenine sulphate kindly supplied to us by Mr A. Chaston Chapman. The adenine sulphate which was recrystallised several times was obtained by Mr Chapman by hydrolysis from yeast nucleic acid. The adenine was precipitated from the sulphate by neutralising it exactly with ammonia and passing a stream of CO_2. The filtered residue was washed with water, alcohol and ether. The adenine gave Kossel's reaction; when heated quickly it melted at 343° (uncorrected) and yielded a picrate consisting of yellow silky needles which charred at 240°. In accordance with Williams and Seidell's directions 50 mg. of adenine were heated at 100° for five hours with 5 cc. of absolute alcohol in which 10 mg. of metallic sodium were dissolved. The orange coloured liquid was then evaporated to dryness on a water-bath and the residue dissolved in distilled water immediately before injection. The following protocol describes five experiments.

Expt. 9. Pigeon 256, which showed well-developed symptoms of polyneuritis with retracted neck, received 10 mg. of untreated adenine; it became worse next day and was found dead on the morning of the third day.

Expt. 10. Pigeon 187 showed paralysed limbs. It received at 4 p.m. the entire residue from 50 mg. of adenine treated as described above. Slight improvement was noticeable on the next day when it again received the residue from 50 mg. of adenine. No change occurred on the third and fourth days; it became worse on the fifth day and remained in the same state during the sixth. On the seventh day it became decidedly worse and was treated with the residue from 50 mg. of adenine. No improvement was observed and the bird died during the night.

Expt. 11. Pigeon 247 showed well-declared symptoms of polyneuritis with retracted neck. It received at 4 p.m. 10 mg. from residue obtained by treating 50 mg. of adenine. The bird was found dead next morning.

Expt. 12. Pigeon 198, which was paralysed but kept its head well forward, received 5 mg. of treated adenine. On the next day it was a little better and received another 5 mg. of the adenine preparation of the previous day in the morning and further 5 mg. of a fresh preparation in the afternoon. On the third day the condition was unchanged and the remaining portion of the 50 mg. of adenine, treated on the previous day, was administered at 10.30 a.m. The bird became worse and died at 2 p.m. the same day.

Expt. 13. Pigeon 217 showed incipient signs of polyneuritis. It received 50 mg. of adenine treated with sodium ethylate. It was better the next day, but had a relapse on the third day and received a further 50 mg. of treated adenine. No improvement ensued and the bird was found dead on the fourth morning.

The above experiments like those performed with α-hydroxypyridine must be regarded as negative in character. In Expt. 10 the bird remained alive for six days, but the condition of the bird during that time was such that nothing could be definitely ascribed to the action of the administered adenine. The results obtained in the remaining experiments are so distinctly negative that no comment is necessary.

Summary.

1. The authors can confirm the following facts observed by Williams about the nature of α-hydroxypyridine.

(*a*) α-Hydroxypyridine obtained by distilling hydroxynicotinic acid crystallises in needles and non-acicular crystals.

(*b*) On keeping the needles of α-hydroxypyridine, thus obtained, exposed under ordinary laboratory atmospheric conditions, they break up into granules in about 10–14 days.

(*c*) Needles of α-hydroxypyridine are also obtained by heating the freshly distilled substance for several hours at 120° and then adding ligroin to a solution of the cooled substance in benzene.

2. The authors find that the needles obtained from benzene-ligroin differ in their melting point from those obtained by direct distillation of α-hydroxypyridine.

3. Both varieties of needles of α-hydroxypyridine were injected into

polyneuritic pigeons but no cure or amelioration in the condition of the birds was observed. The observations of Williams were therefore not confirmed.

4. Crystals obtained by recrystallising α-hydroxypyridine from water proved also to be inactive.

5. Pure adenine, as well as adenine treated with sodium ethylate in a sealed tube for five hours at 100°, yielded negative results in agreement with Voegtlin and White but in disagreement with Williams and Seidell.

REFERENCES.

Pechmann (1891). *Annalen.* **264**, 272.
Pechmann and Baltzer (1891). *Ber.* **24**, 3144.
Pechmann and Welsh (1884). *Ber.* **17**, 2384.
Voegtlin and White (1916). *J. Pharm. Exp. Ther.* **9**, 155
Williams (1916). *J. Biol. Chem.* **25**, 437.
—— (1917). *J. Biol. Chem.* **29**, 495.
Williams and Seidell (1916). *J. Biol. Chem.* **26**, 431

XVIII. COLOUR CHANGES PRODUCED BY TWO GROUPS OF BACTERIA UPON CASEINOGEN AND CERTAIN AMINO-ACIDS.

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The reactions which are described in this paper were undertaken in the course of work which was carried out in an endeavour to discover the causes of discolouration in Stilton cheese. This discolouration which varies from pink to black through many shades of yellow and brown is very widespread, and is a source of serious loss to some Stilton cheese manufacturers. The particular line of work adopted was based on the facts that Beijerinck [1911] had shown that some organisms were capable of producing discolouration in a medium which he had prepared and which contained tyrosine, and that Golding [1912] had, more recently, shown that certain organisms which he had isolated from discoloured cheeses were also capable of producing discolouration on Beijerinck's medium. Sixty-two discoloured Stilton cheeses were procured from a factory, and organisms were isolated from them.

In the very early days of the work the material was plated upon Beijerinck's medium. Although some of the colonies gave well-marked discolouration it was found very difficult to recover the organisms from these colonies. It was thought, therefore, that Beijerinck's medium was too severe, and that there was at least a possibility that colour producing organisms existing in cheeses were being lost. The discoloured cheeses were therefore plated upon + 10, neutral and − 5 lemco agar, Beijerinck's medium and a cheese medium obtained by mixing 20 % cheese and 2 % agar, made neutral and − 5 to Eyre's standard. In this way it was hoped that the organisms of more vigorous growth which could act upon tyrosine would be found upon the Beijerinck plates but the others which were either of less vigorous growth or

required other fractions than tyrosine would be found either upon the agar or cheese media. The reaction was varied because it had appeared that this affected the capacity for colour production. All those colonies which showed colour change on any of these media, + 10, neutral and -- 5 lemco agar, − 1 and − 3 Beijerinck's medium, or neutral and − 5 cheese agar, were reisolated and when pure strains had been obtained were once more tested upon the media mentioned before further work was undertaken.

Bacteriological examination of the pure strains which showed discolouration was then made, with the result that organisms belonging to many different groups were isolated, including *B. coli*, *B. proteus*, *Streptotricheae*, Moulds etc. The present paper deals with strains belonging to only two of these numerous groups, namely *B. proteus* and a group of bacilli (called Gram-negative alkaline), which gave a negative reaction with Gram's stain and produced an alkaline reaction on all the fourteen sugars, alcohols and glucosides into which they were inoculated.

Group 1. *B. proteus vulgaris.*

Thirty-six members of this group were isolated from seven cheeses. Twenty-four strains from these seven were examined bacteriologically and were all found to correspond one with another. From among these, ten from seven cheeses were arbitrarily selected, and were used for further inoculations. Seven strains belonging to this group were isolated from two out of thirty-six samples of milk. These all gave identical reactions. One strain from each sample was taken for further experiment. All the members of this group gave the following reactions. Upon agar plates a rapidly spreading growth occurred. When examined under the microscope their length was seen to vary from 1 to 3μ and a tendency to chain formation was observed. Sometimes very long forms up to 14μ without any apparent division into cells were present. They were Gram-negative and motile, liquefied gelatin, produced indole from peptone water, gave a negative Voges-Proskauer reaction, and produced acid and gas in glucose, saccharose and galactose. The production of acid and gas in salicin, glycerol and dextrin was irregular, while there was no gas production in lactose, mannitol, sorbitol, dulcitol, adonitol, inositol, inulin or raffinose. In litmus milk a soft bleached clot and whey, a bad smell and an alkaline reaction were produced. It was felt that the consistency of these reactions justified the assumption that all these strains were members of one group of organisms and that this group was *B. proteus vulgaris*. Since these organisms produced a reddish brown

colour upon neutral lemco agar and upon the cheese medium which had been made, it was felt that a study ought to be made of their reactions upon caseinogen and its decomposition products. The inoculations were made upon the following media. Caseinogen water, 0·2 % suspension of Griffin's "extra pure casein" in distilled water, the solid being distributed as evenly as possible by continuous shaking during the process of filling out. Tyrosine, tryptophan, alanine, cystine and histidine hydrochloride, 0·1 % solutions in distilled water. Histidine water 0·1 %, the hydrochloric acid being neutralised by the calculated quantity of sodium carbonate, and neutral alanine water 0·1 % in which the carboxyl group was neutralised by the calculated quantity of sodium carbonate. The agars were in all cases made like Beijerinck's medium but contained tyrosine, tryptophan, alanine, cystine and histidine hydrochloride, and in every case were made − 1 and − 3 to Eyre's standard.

The inoculations were all made by transference of a loopful of the culture from neutral lemco agar media. It was thought possible that if colour changes occurred they might be due to the action of the organisms upon traces of agar transferred with the culture. Control experiments in distilled water and saline did not lead to the belief that any serious source of error was introduced in this way. The further possibility that the transference of particles of the agar medium along with the cultures to the amino-acids may have stimulated their growth [Bainbridge, 1911], and thus enabled the organisms to produce the colour changes which were seen, has not been guarded against. All tubes after inoculation were incubated at 30° for one week, then kept in the dark at laboratory temperature. They were examined at the end of 48 hours, 7 days, 14 days, 1, 2 and 3 months and the colour changes produced were recorded. No attempt was made to demonstrate the presence or absence of growth in the tubes.

Tyrosine has long been known to be present in old cheeses, and, with the very kind assistance of Professor F. G. Hopkins, Cambridge, the presence of tryptophan and histidine (or its base) in old Stilton cheeses had been demonstrated. Cystine and alanine are straight chain compounds and were chosen for this work because it had been suggested that it would be impossible to obtain any colouration upon aliphatic substances.

Some striking results were obtained which are shown in Table I.

The results obtained were, on the whole, very consistent for the members of this group and were most striking on tryptophan, in which an amber colour was invariably produced. Upon histidine hydrochloride water there was no visible reaction, but in the neutralised medium a light pinkish brown

colour occurred. Upon tyrosine water there was practically no change, but upon tyrosine agar a discolouration was seen varying from very slight to claret. Upon the other media and the controls no discolouration was produced.

TABLE 1.

No.	Strain	Histidine hydrochloride water	Histidine water	−1 Histidine agar	−3 Histidine agar	Cystine water	−1 Cystine agar	−3 Cystine agar	Tryptophan water	−1 Tryptophan agar	−3 Tryptophan agar
1	$C_{20}2B_2$	–	L. P. B.	v. v. sl.	v. sl.	–	–	–	Amber	L. Amber	L. Amber
2	$C_{22}2B$	–	L. P. B.	v. sl.	v. sl.	–	–	–	,,	,,	,,
3	$C_{22}9B$	–	–	v. L. P.	v. L. P.	–	–	–	,,	Amber	Amber
4	$C_{26}4A^7$	–	L. P. B.	v. L. P.	v. L. P.	–	–	–	,,	,,	,,
5	$C_{26}4A^8$	–	L. P. B.	v. L. P.	v. L. P.	–	–	–	,,	,,	,,
6	$C_{26}4B_1$	–	L. P. B.	v. L. P.	v. L. P.	–	–	–	,,	,,	,,
7	$C_{25}5A$	–	L. P. B.	v. L. P.	v. L. P.	–	–	–	,,	,,	,,
8	$C_{29}9A$	–	L. P. B.	–	v. sl.	–	–	–	,,	,,	,,
9	C_{32}^{3}	–	L. P. B.	+sl.	+sl.	–	–	–	,,	,,	,,
10	$C_{58}1A$	–	v. L. P. B.	v. v. sl.	v. v. sl.	–	–	–	,,	,,	,,
11	$M_{20}1C$	–	L. P. B.	v. L. P.	v. L. P.	–	–	–	,,	,,	,,
12	$M_{26}1C$	–	L. P. B.	v. L. P.	v. L. P.	–	–	–	,,	,,	,,

No.	Strain	Alanine water	Neutral alanine water	−1 Alanine agar	−3 Alanine agar	Caseinogen water	Tyrosine water	−1 Tyrosine agar	−3 Tyrosine agar	Sterile water	Sterile saline
1	$C_{20}2B_2$	–	–	–	–	–	v. sl.	+sl.	+sl.	–	–
2	$C_{22}2B$	–	–	–	–	–	v. v. sl.	Claret	Claret	–	–
3	$C_{22}9B$	v. v. sl.	–	–	–	–	–	+sl.	+sl.	–	–
4	$C_{26}4A^7$	? +	–	–	–	–	–	+sl.	v. sl.	–	–
5	$C_{26}4A^8$	? +	–	–	–	–	–	+sl.	v. sl.	–	–
6	$C_{26}4B_1$	–	–	–	–	–	–	+sl.	+sl.	–	–
7	$C_{25}5A$	–	–	–	–	–	–	+sl.	+sl.	–	–
8	$C_{29}9A$	–	–	–	–	–	–	+sl.	+sl.	–	–
9	C_{32}^{3}	–	–	–	–	–	v. sl.	+sl.	+sl.	–	–
10	$C_{58}1A$	–	–	–	–	–	+sl.	+sl.	+sl.	–	–
11	$M_{20}1C$	–	–	–	–	–	?+	+sl.	+sl.	–	–
12	$M_{26}1C$	–	–	–	–	–	–	sl. Claret	sl. Claret	–	–

L. P B. = Light Pinkish Brown. v. L. P. = Very Light Pink. sl. = slight.

GROUP 2. *Gram-negative, alkaline Bacilli.*

Eleven strains of this organism were isolated from four of the discoloured cheeses which had been obtained from the factory. Seven from four cheeses were studied in detail; twenty-six strains were isolated from twenty-one samples of milk which were being used in the factory. Seventeen from ten samples were examined in detail, and five from five samples were selected arbitrarily for further studies of colour changes.

These organisms all showed the following characteristics; a greenish yellow growth on neutralised potato and complete absence of growth on

lemco agar under anaerobic conditions. In litmus milk after fourteen days' incubation at 30° a flesh-coloured clot, whey, a brown ring at the surface and an alkaline reaction were found. When examined under the microscope they appeared as somewhat exaggerated *B. coli*; they were Gram-negative, motile, gave the indole reaction with peptone water, and a negative Voges-Proskauer reaction; they liquefied lemco gelatin with the production of a brownish colour when the medium was neutralised. Upon glucose, lactose, saccharose, galactose, raffinose, dulcitol, inositol, sorbitol, mannitol, adonitol, inulin, salicin, dextrin and glycerol they gave a consistently alkaline reaction. Fifty-four samples of water taken from the farms which were supplying milk to the factory were examined. Of these, eighteen showed organisms belonging to this group. Seventeen strains from seventeen samples have been further examined. Six of these strains, W_2^{E}, W_{17}^{1A}, W_{19}^{1B}, W_{26}^{2}, $W_{32}^{3B,A}$ and $W_{50}^{1A^1}$, selected arbitrarily, showed all those reactions which have been already described for the cheese and milk strains. The remainder, $W_{14}^{1A^1}$, W_{21}^{1}, W_{25}^{3A}, W_{27}^{2}, W_{29}^{1}, W_{30}^{2B}, W_{31}^{1A}, W_{36}^{1C}, W_{42}^{3B}, $W_{45}^{1A_2}$ and W_{52}^{3A} were not tested upon galactose, raffinose, inositol, sorbitol, mannitol, adonitol, inulin, salicin, dextrin or glycerol, because Kahlbaum's preparations were being used and it was necessary to conserve them. In all other respects their reactions were identical with those which have been described for the other strains.

The twenty-nine strains which were selected comprised seven from four cheeses, five from five samples of milk and seventeen from seventeen samples of water. The colour changes resulting from the action of these organisms upon various media which have been already described under Group 1 are shown in Table II.

The results show that, when inoculated into histidine hydrochloride water, the reactions were either definitely negative or very doubtfully positive, and on only one occasion was a very light brown colour produced. When the hydrochloric acid of the histidine hydrochloride was neutralised, the colour was slightly more marked, though even then the maximum colour recorded is slight brown. Upon the histidine agar media the results were, for all practical purposes, negative. Negative results were also obtained with all the cystine media.

Upon the tryptophan water and agar media the results were very consistent, being much less marked upon the agar than upon the tryptophan water, upon which they showed a colour varying from slight yellow to a definite canary. Upon alanine again, the results on the agar media were

TABLE II.

No.	Strain	Histidine hydrochloride water	Histidine water	−1 Histidine agar	−3 Histidine agar	Cystine water	−1 Cystine agar	−3 Cystine agar	Tryptophan water	−1 Tryptophan agar	−3 Tryptophan agar	Alanine water
1	$C_{22}{}^{4}B$	–	v. sl. Y.	–	–	–	–	–	sl. Y.	+v. sl.	+v. sl.	sl. Y.
2	$C_{23}{}^{9}$	–	v. sl. Y.	–	–	–	–	–	sl. Y.	+v. sl.	+v. sl.	v. sl. Y.
3	$C_{25}{}^{5}C_{1}$	?+	sl. Y. B.	–	–	–	–	–	sl. Y.	+v. sl.	+v. sl.	sl. Y.
4	$C_{26}{}^{5}C_{2}$	?+	+sl.	–	–	–	–	–	sl. Y.	+v. sl.	+v. sl.	v. sl. Y.
5	$C_{27}{}^{1}A$	+sl.	+sl.	–	–	–	–	–	Canary	+sl.	+sl.	v. sl. Y.
6	$C_{27}{}^{5}C_{4}$	?+	sl. B.	–	–	–	–	–	,,	+sl.	+sl.	v. sl. Y.
7	$C_{27}{}^{9}A$	–	?+	–	–	–	–	–	,,	+v. sl.	+v. sl.	v. sl. Y.
8	$M_{3}{}^{2}A$	?+	+sl.	–	–	–	–	–	,,	+sl.	+sl.	sl. Y.
9	$M_{6}{}^{1}$	–	+sl.	–	–	–	–	–	,,	+sl.	+sl.	v. sl. Y.
10	$M_{28}{}^{1}A$	–	+v. sl.	–	–	–	–	–	v. sl. Y.	+v. sl.	+v. sl.	sl. Y.
11	$M_{29}{}^{3}A$	?+	+sl.	–	–	–	–	–	Canary	+v. sl.	+v. sl.	sl. Y.
12	$M_{32}{}^{3}A$	+v. sl.	+v. sl.	–	–	–	–	–	,,	+sl.	+sl.	sl. Y.
13	$W_{2}E$	–	sl. B.	–	–	–	?+	?+	,,	+v. sl.	+v. sl.	sl. Y.
14	$W_{14}{}^{1}A_{1}$	–	+sl.	–	–	–	–	–	L. Canary	+v. sl.	+sl.	sl. Y.
15	$W_{17}{}^{1}A$	–	?+	–	–	–	–	–	Canary	+sl.	+sl.	sl. Y.
16	$W_{19}{}^{1}B$	?+	?+	–	–	–	–	–	L. Canary	+sl.	+sl.	sl. Y.
17	$W_{21}{}^{1}$	–	+sl.	–	–	–	–	–	,,	+v. sl.	+v. sl.	+v. sl.
18	$W_{25}{}^{3}A$	v. L. B.	+v. sl.	–	?+	–	–	–	,,	+sl.	+sl.	?+
19	$W_{26}{}^{2}$	?+	?+	–	–	–	?+	?+	Canary	+sl.	+sl.	sl. Y.
20	$W_{27}{}^{2}$	–	+sl.	–	–	–	–	–	,,	+sl.	+sl.	sl. Y.
21	$W_{29}{}^{1}$	–	L. B.	–	–	–	–	–	L. Canary	+v. sl.	+v. sl.	sl. Y.
22	$W_{30}{}^{2}B$	?+	v. L. B.	–	–	–	–	–	,,	+v. sl.	+v. sl.	?+
23	$W_{31}{}^{1}A$	–	?+	–	–	–	–	–	,,	+sl.	+sl.	?+
24	$W_{32}{}^{3}B_{1}A$	+v. v. sl.	+v. sl.	–	–	–	–	–	,,	+sl.	+sl.	sl. Y.
25	$W_{36}{}^{1}C$	–	sl. B.	–	–	–	–	–	,,	+sl.	+sl.	?+
26	$W_{42}{}^{3}B$	–	v. L. B.	–	–	–	–	–	,,	+sl.	+sl.	sl. Y.
27	$W_{45}{}^{1}A_{2}$	–	+sl.	–	–	–	–	–	,,	+sl.	+sl.	+v. sl.
28	$W_{50}{}^{1}A_{1}$	?+	+sl.	–	–	–	–	–	Canary	+sl.	+sl.	+v. sl.
29	$W_{52}{}^{3}A$	–	+sl.	–	–	–	–	–	,,	+sl.	+sl.	+v. sl.

No.	Strain	Neutral alanine water	−1 Alanine agar	−3 Alanine agar	Caseinogen water	Tyrosine water	−1 Tyrosine agar	−3 Tyrosine agar	Sterile water	Sterile saline
1	$C_{22}{}^{4}B$	sl. Y.	+v. v. sl.	+v. v. sl.	Brown	Bl.	Bl.	Bl.	–	–
2	$C_{23}{}^{9}$	sl. Y.	?+	,,	,,	B.—Bl.	Bl.	Bl.	–	–
3	$C_{25}{}^{5}C_{1}$	sl. Y.	+v. v. sl.	,,	,,	B.—Bl.	Bl.	Bl.	?+	?+
4	$C_{26}{}^{5}C_{2}$	sl. Y.	,,	,,	,,	B.—Bl.	Bl.	Bl.		
5	$C_{27}{}^{1}A$	sl. Y.	,,	,,	,,	B.—Bl.	Bl.	Bl.	?+	–
6	$C_{27}{}^{5}C_{4}$	+sl.	,,	,,	,,	B.—Bl.	Bl.	Bl.		
7	$C_{27}{}^{9}A$	sl. Y.	,,	,,	,,	B.—Bl.	Bl.	Bl.	–	–
8	$M_{3}{}^{2}A$	sl. Y.	,,	,,	,,	B.—Bl.	Bl.	Bl.	–	–
9	$M_{6}{}^{1}$	v. sl. Y.	,,	,,	,,	D. B.	Bl.	Bl.		
10	$M_{28}{}^{1}A$	+v. sl.	,,	,,	L. Brown	B.—Bl.	Bl.	Bl.	–	–
11	$M_{29}{}^{3}A$	sl. Y.	,,	,,	Brown	B.—Bl.	Bl.	Bl.		
12	$M_{32}{}^{3}A$	sl. Y.	,,	,,	,,	B.—Bl.	Bl.	Bl.		
13	$W_{2}E$	sl. Y.	,,	,,	v. L. Brown	B.	Bl.	Bl.		
14	$W_{14}{}^{1}A_{1}$	sl. Y.	,,	,,	Brown	B.—Bl.	B.—Bl.	B.—Bl.		
15	$W_{17}{}^{1}A$	v. sl. Y.	,,	,,	L. Brown	D. B.	Bl.	Bl.		
16	$W_{19}{}^{1}B$	v. sl. Y.	,,	,,	,,	B.—Bl.	Bl.	Bl.		
17	$W_{21}{}^{1}$	v. sl. Y.	,,	,,	,,	B.—Bl.	B.	Bl.		
18	$W_{25}{}^{3}A$	sl. Y.	,,	,,	Brown	B.—Bl.	B.—Bl.	Bl.		
19	$W_{26}{}^{2}$	sl. Y.	,,	,,	L. Brown	B.—Bl.	Bl.	Bl.		
20	$W_{27}{}^{2}$	sl. Y.	,,	,,	Brown	D. B.	B.	B.—Bl.		
21	$W_{29}{}^{1}$	sl. Y.	,,	,,	,,	B.—Bl.	B.—Bl.	Bl.	–	
22	$W_{30}{}^{2}B$	sl. Y.	,,	,,	,,	B.—Bl.	B.	B.—Bl.		
23	$W_{31}{}^{1}A$	sl. Y.	,,	,,	,,	B.—Bl.	L. B.	Bl.		
24	$W_{32}{}^{3}B_{1}A$	+v. sl.	,,	,,	,,	B.—Bl.	B.—Bl.	Bl.	?+	
25	$W_{36}{}^{1}C$	?+	,,	,,	,,	B.—Bl.	B.—Bl.	B.—Bl.		
26	$W_{42}{}^{3}B$	sl. Y.	,,	,,	,,	B.—Bl.	B.—Bl.	Bl.		
27	$W_{45}{}^{1}A_{2}$	+v. sl.	,,	,,	L. Brown	B.—Bl.	Bl.	Bl.		
28	$W_{50}{}^{1}A_{1}$	+v. sl.	,,	,,	Brown	B.—Bl.	Bl.	B.—Bl.	?+	–
29	$W_{52}{}^{3}A$	v. sl. Y.	,,	,,	,,	B.—Bl.	Bl.	B.		

L. = Light. D. = Dark. sl. = Slight. Y. = Yellow. B. = Brown. Bl. = Black.

less marked than upon the water. The results upon alanine water and upon neutralised alanine water did not appear to differ greatly, the maximum effect obtained being a slight yellowish colour. Upon caseinogen water, these organisms again produced a definite brownish colour, which differentiated them completely from the *proteus* group, the members of which failed entirely to produce any change upon this medium. Upon the tyrosine media the colours varied from brown to black. The control tubes made by the inoculation of sterile saline and sterile water were, as a rule, quite negative, but occasionally showed a very doubtful colour change.

The maximum and minimum reactions for each group are shown in Table III.

TABLE III.

No.	Strain	Histidine hydrochloride water	Histidine water	−1 Histidine agar	−3 Histidine agar	Cystine water	−1 Cystine agar	−3 Cystine agar	Tryptophan water	−1 Tryptophan agar	−3 Tryptophan agar
1	*B. proteus vulgaris*	– to v. sl.	v. L. Y. to P. B.	– to L. P.	v. v. sl. to L. P.	– to +sl.	– to ?+	– to ?+	L. Amber to D. Amber	L. Amber to D. Amber	L. Amber to D. Amber
2	Gram-neg. alkaline	– to ?+	?+ to L. B.	– to v. sl.	– to +sl.	– to v. sl.	– to v. sl.	– to v. sl.	v. sl. Y. to Canary	v. sl. to +sl.	v. v. sl. to +sl.

No.	Strain	Alanine water	Neutral alanine water	−1 Alanine agar	−3 Alanine agar	Caseinogen water	Tyrosine water	−1 Tyrosine agar	−3 Tyrosine agar	Sterile water	Sterile saline
1	*B. proteus vulgaris*	– to v. v. s.	–	–	–	–	– to L. B.	+sl. to Claret	v. sl. to Claret	–	–
2	Gram-neg. alkaline	v. sl. to sl. Y.	– to sl. Y.	?+ to v. v. sl.	v. v. sl.	v. sl. to D. B.	B. to Bl.	L. B. to Bl.	B. to Bl.	– to ?+	– to ?+

B. = Brown. Bl. = Black. D. = Dark. L. = Light. P. = Pink. Y. = Yellow. sl. = Slight.

The most characteristic differences between the members of these groups under the particular conditions of experiment, are the production of a well-marked amber colour upon tryptophan and absence of reaction in caseinogen water by members of the *proteus* group, with the production of a colour which was never more than canary yellow upon tryptophan and of a definite brown colour in caseinogen water by members of the Gram-negative, alkaline group.

SUMMARY.

A very large number of organisms belonging to many different groups have been isolated from discoloured Stilton cheeses, milk from which such cheeses were made and the water supplies of the farms from which the milk was obtained.

The bacteriological reactions of two of these groups have been studied.

Certain members of these two groups have been selected arbitrarily and their reactions upon caseinogen and various amino-acids resulting from its degradation have been investigated.

The results show that members of both groups are capable, under the conditions of experiment, of producing discolouration upon certain of the substances selected and that the two groups show very distinct differences in their capacity for producing colour upon the various media upon which they were tested.

REFERENCES.

Bainbridge (1911). *J. Hygiene*, **11**, 341.
Beijerinck (1911). *K. Akad. Wetensch. Amsterdam*, **19**, 1092.
Golding (1912). *J. Board of Agric.* **19**, No. 3.